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Expression Profile Analysis of Early Development and Gravity Response of Germinating *Ceratopteris richardii* Spores

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Expression Profile Analysis of Early Development and Gravity Response of Germinating *Ceratopteris richardii* Spores

by

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Expression Profile Analysis of Early Development and Gravity Response of Germinating *Ceratopteris richardii* Spores

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Single-celled germinating spores of the fern *Ceratopteris richardii* possess a mechanism to detect the directional force of gravity, and they respond to this force by establishing the axis of their development in the direction of the gravity vector prior to their first cell division. In this dissertation, I describe the construction of cDNA microarrays containing almost 3000 different genes expressed in germinating *Ceratopteris* spores 20 h after they are induced to germinate by light, just after gravity has established the polarity of their growth. Using these microarrays, I have analyzed gene expression changes that occur over time during early development and while the spores were being subjected to the

microgravity environment of spaceflight on Shuttle mission STS93. Selected changes detected by microarray analysis were verified by quantitative real-time RT-PCR.

In the early development study I used microarrays to evaluate the expression profile of spores during the first 48 h of development as they break dormancy, respond to gravity, and prepare for the first cell division. Spore-expressed genes have been compared to the genes uniquely expressed in two other germinating systems, *Arabidopsis* seeds and *Arabidopsis* pollen. My results provide novel data on genes expressed during spore germination, and thus give new insights into the molecular mechanism by which plant cells accomplish the important process of emerging from their dormant phase.

This dissertation also presents the first microarray analysis of the changes in gene expression induced by spaceflight in a plant system. Spores flown on NASA flight STS-93 were compared to ground-control samples. Results are discussed in relation to both gene expression changes induced by gravity on earth and to physiological changes observed in other plant systems as a response to microgravity. They are consistent with a model for early cellular responses to gravity perception that include a cytoplasmic pH change and/or calcium mediated polar vesicle transport.

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CHAPTER 1

INTRODUCTION

The gametophyte generation of the homosporous aquatic fern *Ceratopteris richardii* is a valuable model system for the study of many physiological processes in plant growth and development (Chatterjee and Roux, 2000). The short generation time (about three months), relative simplicity of the mature gametophyte plant, and ease of propagation in the lab have made *Ceratopteris* a useful model for the study such diverse processes as sex determination and differentiation (Banks et al., 1999), hormone induction of lateral root formation (Blancaflor et al., 2004), photomorphogenesis through mutant analysis (Kamachi et al., 2004), and gravity-directed polar development (Edward and Roux, 1998; Chatterjee et al., 2000).

In this dissertation I have increased the value of *Ceratopteris* as a model system by contributing a detailed microarray assessment of the gene expression changes that occur, both at 1-*g* and in microgravity, among over 3000 different genes expressed in *Ceratopteris* during the earliest stages of gametophyte development after the spore has been induced to germinate by light. This includes the period during which gravity fixes the polarity of the cell, so the results permit a unique

assessment of how the subtle force of gravity impacts gene expression in a single-cell system. Also, because this is the first published microarray analysis of gene expression during the germination of fern spores, it provides a unique opportunity to compare this expression pattern with those previously described in angiosperms for distinctly different cell and tissue types that are also emerging from dormancy.

Gametophyte Early Development and Germination

Ceratopteris spores are the resistant state of the haploid gametophyte that remain dormant until imbibed and induced to germinate by exposure to light. Spore germination is a red-light induced, far-red light inhibited photo-reversible process (Cooke et al., 1987). Upon exposure to white or red light, spores begin a series of developmental steps that I will refer to as gametophyte early development, that result in germination as defined by emergence of the primary rhizoid of the gametophyte. The first physiological event documented in the gametophyte's early development is an efflux of calcium from the top of the spore, which is concurrent with an influx of calcium at its bottom (Chatterjee et al., 2000). This calcium current is parallel to the vector of gravity and re-orients as quickly as the detection system can be repositioned when the spore is rotated 180 degrees. The calcium current predicts the direction of the migration of the spore's cell nucleus, which

occurs 24–30 h after the spore is initially exposed to light. The direction that this nuclear migration will take is determined 6 to 20 h prior to its occurrence (Edwards and Roux, 1998). This indicates that within the first 18–24 h of development the spore has responded to the force of gravity by irreversibly fixing the polarity of its development. Following the nuclear migration 24 h after light exposure, there is an asymmetric cell division that results in a smaller cell that will develop into the primary rhizoid of the gametophyte, and a larger cell that will develop into the photosynthetic thallus (Raghavan, 1989).

The spore of *Ceratopteris* is biologically analogous to the microspore or megaspore of an angiosperm, being the single cell stage of the gametophyte generation. Fern spores, however, may be more similar physiologically to the seed stage of an angiosperm. Both seeds and spores are a quiescent dormant stage of the plant life cycle that allows dispersal and resistance to unfavorable growing conditions. Both emerge from their dormancy as a result of environmental cues that indicate conditions are favorable, and develop into free living photosynthetic plants. Angiosperm seeds consist of several cell and tissue types, both maternal and embryonic in origin, and are more complex in structure than the single celled spore of *Ceratopteris*. For this reason, *Ceratopteris* spores are a simpler model for study of the emergence from dormancy and early development of plants.

Ceratopteris spores are semi-aquatic in their natural habitat and thus will germinate and develop when sown on agar and growth media in humid conditions. The primary rhizoid emerges from the spore 72–96 h after light exposure and, in over 90% of spores placed in a fixed orientation, when the primary rhizoid emerges it grows downward parallel to the vector of gravity (Edwards and Roux, 1998). Analysis of spore development in microgravity on NASA shuttle flight STS-93 revealed that polar development occurred in the absence of gravity, including polar nuclear migration (which determines the direction of primary rhizoid growth), but it was randomly oriented (Roux et al., 2003).

In plants, gravity perception and response are often studied in the gravitropic, multicellular root or shoot of an angiosperm (Chen et al., 2002; Yano et al., 2003; and Johannes et al., 2001). The mechanism of perception and the growth changes in response to the signal of gravity may not occur in the same cells in these systems. This fern spore is a single cell that possesses a mechanism to sense the force of gravity and respond to it with a visible physiological change, thereby eliminating the complication of intercellular communication of the signal.

DNA Microarray

Environmental stimuli often induce differential expression of genes that mediate the adaptive responses to those stimuli. For example, heat-

shock proteins are up-regulated in response to increased temperature (Keeler et al., 2000), the expression of several calmodulin genes is altered in response to touch (Botella and Arteca, 1994), and genes related to photosynthesis are up-regulated by light (Lopez-Juez et al., 1998). In these examples and others, genes whose expression is altered by a stimulus play a key role in the signal transduction and physiological response to that stimulus. DNA microarray technology has provided a method of monitoring expression profiles, in any experimental condition, of thousands of genes. In plants, microarray analysis has been used to study changes during floral development and development of reproductive structures (Hennig et al., 2004), growth under conditions of biotic and abiotic stress (Schenk et al., 2000; see review Reymond, 2001) or salt stress (Marin et al., 2003), and how gene expression is altered in mutants (Mandaokar et al., 2003).

Microarray analyses of gene expression changes that occur in germinating fern spores during the period when gravity is directing their polarity would provide valuable insights into what genes may be critical for this process, and Chapters 2 and 3 provide two different versions of this information. Initiating a project to assess gene expression changes early during the process of spore germination begs the question of whether new gene transcription is needed to direct this process, or whether all the

transcripts needed are already present in the dormant spore. To investigate this question we tested the effect of inhibiting transcription on the spore germination rate. The results (Fig. 2.1) showed that new RNA is indeed needed for spores to germinate.

There are two basic types of DNA microarray, oligonucleotide arrays and cDNA arrays. Both can be used for similar types of analysis, including gene expression monitoring, but they differ in construction and design. Both types of microarray allow the user to identify quantitative differences in transcript abundance between two samples for all features, or genes, represented on the array.

DNA microarray technology is based on the principle that single strands of nucleic acid will anneal, or hybridize, with complementary strands. In all microarray production, some form of a gene, either amplified cDNA or an oligonucleotide, is laid down on a solid surface, these being the “features” on the array. The number of features that can be included in an array varies, depending on the method of construction. These features are the genes that are being simultaneously monitored for changes in expression in a hybridization experiment.

In cDNA microarray analysis, to obtain differential expression data, fluorescent probes synthesized from a defined pool of expressed RNA are applied to the array to allow complementary sequences in the fluorescent

probe to hybridize with the features on the array. Microarray expression data is a comparison between two pools of RNA, for instance RNA isolated from leaves of *Arabidopsis* plants grown in normal light conditions and from leaves of plants grown in high light conditions (Rossel et al., 2002), with the two different pools labeled with two different colors of fluorescent dyes, green and red, respectively. The abundance of each feature on the array can be measured by laser scanner for each of the treatment samples (represented by different colors of fluorescent probe) to determine the relative difference in abundance of each gene on the array.

Synthetic oligonucleotide arrays are produced by several private companies (Affymetrix being the best known currently) and can be purchased as a mass-produced catalog array, such as the Affymetrix GeneChip®, or as a custom designed array. These arrays can contain over one million "probes" which are generally 25-mer oligonucleotides (in the case of Affymetrix-produced chips) that are produced on a glass chip through a patented process of light-directed synthesis, or photolithography (Lipshutz et al., 1999).

The features of the oligonucleotide arrays are produced and catalogued based on gene sequence information, and are completely dependent on the annotation of genes available for the organism. This means that "genes" represented on the array may not be expressed at all,

but may be pseudogenes incorrectly identified in a large scale sequencing effort. Another consideration with commercially produced oligonucleotide arrays is dependence on the company that produced them for array scanning hardware and scan analysis software. Although many array analysis programs are available for public use at no cost or can be purchased, the format of data obtained from oligonucleotide array companies is not always compatible with the publicly-available programs. A major advantage of the oligonucleotide system of DNA microarray is that the density of features possible with this type of array construction exceeds all other commonly used array types; therefore, oligonucleotide arrays can be used to measure expression levels of more genes simultaneously, thereby increasing the amount of expression data obtained from each hybridization. Oligonucleotide arrays representative of the entire genome of several plant model organisms, including *Arabidopsis*, soybean and rice are available for purchase from several companies as catalog items at minimal cost ($100-$500 a chip). However, producing custom arrays of other organisms through these companies may be cost prohibitive.

The alternative to synthetic oligonucleotide arrays is a printed cDNA microarray. The construction of this type of microarray requires PCR amplification of the feature genes, then depositing these genes on a

solid surface, usually a microscope slide. Most commonly, cDNA libraries are randomly sampled and individual clones transferred to microtiter plates for partial sequencing to generate expressed sequence tags (ESTs). These clones of known sequence are then amplified by PCR in 96- or 384-well plates followed by cleanup and precipitation of the PCR products. The amplified, purified cDNA is then re-suspended in an appropriate salt buffer, and a high speed arraying machine is used to deposit this cDNA on a glass microscope slide coated with a polymer that allows adherence of the DNA (Schena et al., 1995). Parts lists and instructions for constructing this type of arraying machine are available publicly (http://cmgm.stanford.edu/pbrown/mguide/index.html), and there are numerous different types of scanning hardware and software available. One major advantage printed cDNA microarrys have over synthetic oligonucleotide arrays is the ability to make the arrays, as well as all steps in their production, in-house. This means that, although there is substantial cost associated with initial production and set-up, generating additional arrays requires minimal time and cost.

The features present on a printed cDNA microarray are gene products, and because of the method of obtaining these sequences, something is known about the temporal and physical location of their expression. The method of random sampling of a cDNA library for partial

sequencing and compiling an EST library means that the number of ESTs of the same gene obtained is related to the abundance of that gene in the cDNA library (Van der Hoeven et al., 2002). Often the most abundant genes in the cDNA library will be represented numerous times on a printed cDNA microarray, and this serves as an internal control in the array.

The features printed on a cDNA microarray are typically PCR amplified products of 500 to 3,000 bases in length. The size of feature spots and the distance between spots varies greatly in cDNA microarrays, depending on the arraying machine and printing tips used. Up to 42,000 features can be printed on a cDNA microarray (http://www.dkfz.de/mga/groups.asp?siteID=95), less then half the number of features that oligo-arrays can contain. Printed cDNA microarrays from several different organisms as well as cDNA clone sets that can be purchased are currently being produced by numerous companies and academic groups. There are also companies and groups that will produce custom-printed cDNA microarrays from any cDNA clone set. In addition to these, oligonucleotide clone sets are available that represent the entire genome of a model organism such as *Arabidopsis* or mouse that can be purchased and printed by this method with a high speed arraying machine.

Summary and Overview

In this dissertation I report on the construction of a printed cDNA microarray that contains over 3,000 unique *Ceratopteris richardii* genes, obtained from ESTs of a cDNA library based on RNA isolated from spores 20 h after they were induced to germinate by light. I have used this microarray to study the changes in gene expression that occur during the early development of this single cell, as it breaks dormancy and undergoes the physiological processes described above. It is possible that a dry fern spore may contain all the messages necessary to complete germination, defined as primary rhizoid emergence from the spore coat. The dormant *Ceratopteris* spore contains a significant amount of mRNA, demonstrated by the ability to produce fluorescent first- strand cDNA from spores at the initial time of illumination. Illumination might trigger protein synthesis from the mRNA complement of dormant spores that is sufficient to complete germination. This scenario appears not to be true for *Fucus* zygotes, where the transcription is necessary for rhizoid emergence in the development of zygotes of the brown alga *Fucus* (Quatrano, 1968).

The requirement of new mRNA synthesis in spore germination was verified by experiments much like those described for *Fucus* germination (Quatrano, 1968) in which the spores were treated with the drug actinomycin D that inhibits transcription by blocking RNA polymerase

extension. The germination of spores was delayed by inhibition of transcription, presumably until the light labile actinomycin D used was degraded.

Chapter two of this dissertation describes analysis of spore gene expression during the first 48 h of development. It is a slightly modified version of a research article entitled "Profile and analysis of gene expression changes during early development in germinating spores of *Ceratopteris richardii,"* co-authored by Mari L. Salmi, Thomas J. Bushart, Stephen C. Stout, and Stanley J. Roux that has been accepted for publication in the journal Plant Physiology. Included in this developmental time course is the narrow period of development when the spore senses and responds to gravity. The specific contributions to this work that were made by Thomas Bushart and Dr. Stephen Stout are noted in the Materials and Methods section of chapter two.

Surviving harsh environmental conditions by production of resistant, dormant structures such as seeds or spores is common to all plants. This is the first study of emergence for dormancy and the metabolic processes involved therein by high through-out genomic or proteomic methods. When similar analysis of the changes in gene expression that occur during seed germination become available a comparison to the data

I present here will be valuable in understanding the evolution of plant genes involved in these processes.

We also used this microarray to study the difference in the gene expression profile of spores allowed to germinate in microgravity on shuttle flight STS93 versus spores simultaneously germinating under duplicated conditions in 1-*g* (chapter three). These differences were assayed at three time points early during gametophyte germination. This is the first microarray analysis of plant material flown on a space shuttle. Very little is currently known about the cellular mechanism of plant gravity perception. It is possible that gravity perception and response in cells occurs independent of gene expression changes, however, the approach described here has identified changes in gene expression induced by spaceflight and this may help determine the earliest cellular signaling as a response to gravity.

Until this study there was very little molecular characterization of the genes involved in gravity-directed polar development in plant cells, therefore the genes with expression changes observed in this analysis have been compared to those reported to change during the cell polarizations that accompany development in various plants and animals. Findings from other physiological and cellular studies of the effect of spaceflight and simulated microgravity on plant cells and whole plants have also been

referenced to aid in assessing the significance of the expression changes induced by spaceflight.

The raw data of all microarray analyses and all other supplemental material (including Supplemental Tables) for this dissertation are found at the following URL:

http://www.sbs.utexas.edu/roux/Ceratopteris%20Page/ceratopteris_research.htm

The results described here provide a significant increase in our understanding and knowledge of the earliest genetic changes that occur when plants emerge from dormancy and when they are subjected to the microgravity conditions of spaceflight. In so doing they also reveal genes likely to play critical roles in early gametophytic development and in gravity-directed polar development in *Ceratopteris* and thus provide an important basis for future studies on these topics.

CHAPTER 2

PROFILE AND ANALYSIS OF GENE EXPRESSION CHANGES DURING EARLY DEVELOPMENT IN GERMINATING SPORES OF *CERATOPTERIS RICHARDII*

INTRODUCTION

The ability to survive harsh conditions and emerge from a dormant state and develop into a new organism is a characteristic common to all land plant life cycles. This emergence from dormancy can be seen in the complex processes of angiosperm seed germination, as well as in the germination of pollen, both of which play central roles in determining food production. To study the process of emergence from dormancy and the early stages of development in a germinating system, we are using the spore of the homosporous aquatic fern *Ceratopteris richardii*. This system parallels the simplicity of *Arabidopsis* pollen, where intercellular interactions do not complicate the process, and has a physiological resemblance to many germinating seeds, since it is a phytochrome-mediated response (Cooke et al., 1987) that results in the production of a free living, photosynthetic, multicellular organism.

Ceratopteris has been used to study such diverse processes as sex determination and differentiation (Wen et al., 1999), hormone responses (Hou et al., 2004), photomorphogenesis (Kamachi et al., 2004), and gravity-directed polar development (Edwards and Roux, 1998; Chatterjee et al., 2000). Imbibed spores remain dormant until exposed to light, after which they begin a documented series of developmental steps, including the production of a detectable polar calcium current that peaks 6 h after light exposure (Chatterjee et al., 2000), migration of the nucleus at the end of the first day, a polar cell division one day later, and subsequent primary rhizoid emergence in the direction determined by the nuclear migration at the end of the third day after light exposure. As demonstrated by Edwards and Roux (1998), the spores fix the direction of their nuclear migration and primary rhizoid emergence as a response to gravity in the 6 h to 18 h after light exposure. Here, we provide an initial analysis of the mRNA abundance changes that accompany these physiological changes.

Partial sequencing of cDNA clones as expressed sequence tags (ESTs) is an alternative to more extensive genome sequencing efforts. Large-scale EST sequencing projects (>40,000 ESTs) have been described in a number of plant systems including corn, tomato, moss, soybean, and clover (Fernandes et al., 2002; Van der Hoeven et al., 2002; Rensing et al., 2002; Shoemaker et al., 2002; Sawbridge et al., 2003).When cDNA clones

are randomly selected for sequencing, the abundance of ESTs for the same gene is related to the expression level of that gene in the cDNA library. This relationship enables expression analysis of genes within a sample or comparisons between libraries constructed from biologically distinct samples using only the EST abundance data generated from sequencing and analysis (Fernandes et al., 2002). Additionally, ESTs are useful for other expression analysis techniques, including cDNA microarray, which relies upon known sequence information.

DNA microarray technology has provided a method of monitoring the expression profiles of almost any biological system during developmental changes (Hennig et al., 2004), under conditions of stress response (Marin et al., 2003; Schenk et al., 2000), and as a means of mutant analysis (Mandaokar et al., 2003). To date, microarrays have been used to determine the mRNA complement of *Arabidopsis* seed (Girke et al., 2000) and *Arabidopsis* pollen (Honys and Twell, 2003), but there has been no analysis of the expression changes occurring during development of these systems. This study is aimed at evaluating and documenting the gene expression changes that occur during the emergence from dormancy and early development of a fern gametophyte using thoroughly replicated microarray data analyzed by a Bayesian model for determining statistically significant changes. We present data from 34 array hybridizations that

include at least eight replications of four different developmental time point comparisons. Analysis of these data reveals over 900 genes with relative changes in transcript abundance over the first 48 h of spore development. Several changes in expression observed in microarray analysis have been independently verified by real time quantitative RT-PCR.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

Spores of the fern *Ceratopteris richardii* of an inbred diploid strain designated Hn-n were surface sterilized as described in Edwards et al. (1989). Spores were allowed to soak in sterile water in complete darkness at 29° for 4 to 7 days to enhance synchronization of germination. After soaking, the water was removed, and spores were sown in a solution of half strength Murashige and Skoog Basal Medium (Sigma-Aldrich, St. Louis, MO) pH 6.3 and 0.5% agarose cooled to a temperature below 55°. A concentration of 1 g of spores in 20 mL of media was used in this study, and aliquots of 5 mL were placed into 100 X 15 mm Petri plates, allowed to solidify and maintained in a fixed orientation. Spores were exposed to continuous white light and kept at 29° for varying times as specified. Plates were then sealed and stored at -80° for RNA isolation.

RNA Isolation

Spores frozen on plates in agar were removed and quickly ground to homogeneity with a mortar and pestle. An equal volume (5 mL) of buffer (1M Tris pH 7.3, 5mM EDTA pH 8.0, and 1% SDS) was added with two volumes (10 mL) acidic phenol:chloroform:IAA (Fisher Scientific, Hampton, NH). The mixture was vortexed and distributed into 12 to 18 1.5 mL epi-tubes. One 3.2 mm stainless steel or tungsten bead was added to each tube and a Retsch Mixer Mill 300 was used at top speed (30 oscillations/sec) for five minute intervals two times to disrupt spores. Homogenate was examined under a light microscope to verify that spores were broken open. This mixture was immediately centrifuged at 15000 rpm for 15 min at 15°. The aqueous layer was removed and extracted with chloroform. The aqueous layer was again removed and 1/10 volume of 3M NaAcetate and 2.5 volumes 95% ethanol were added and the solution was allowed to precipitate overnight in -80°. The samples were then centrifuged at 1500 rpm for 15 min, and all pellets from one plate of spores were resuspended and combined in DEPC treated water. Resuspended nucleic acid pellets were then treated with Amplification Grade Deoxyribonuclease I (Invitrogen, Carlsbad, CA) following the manufacturers protocol, and ethanol precipitated as described above. The final RNA pellets were resuspended in 50 µl of DEPC treated water and the

RNA concentration was determined spectrophotometrically. The integrity of the RNA samples was verified by electrophoresis on 1.2% denaturing gels (NorthernMax-Gly denaturing gel; Ambion, Austin, TX).

Spore Treatment with Radiolabeled Nucleotides and Actinomycin D

Spores were sterilized and soaked for synchronization of germination as described. Tritiated dUTP ([5,6-^{3}H] uridine 5'-triphosphate, NH_3 salt in ethanol:H_2O (1:1)) was used in this assay at varying concentrations of between 0.3 and 3.0 μCi/mL. Tritiated dUTP was added to spores at the time they were sown in agarose-containing media.

Experimental samples were treated with various concentrations of between 0.2 and 40 μg/mL of actinomycin D. Stock solution of actinomycin D was dissolved in ethanol, therefore control plates had an equal volume of ethanol added. The actinomycin D was added to the spores at the initial time of illumination along with tritiated dUTP, and kept in continuous light until germination rates were measured at various time-points. For samples used in transcription rate measurements, spores were frozen at various time points. Incorporation of tritiated dUTP was verified by scintillation-counter measurements of 10 μg of extracted total RNA in 3 mL of scintillation fluid and recorded as CPM/μgRNA.

cDNA Library Construction and EST Sequencing

RNA isolated from spores 20 h after light initiation of germination (~24 h before the first cell division) was used for a commercially prepared cDNA library (Life Technologies, Rockville, MD). Randomly chosen clones were sequenced at the Purdue Agricultural Genomics Facility, Purdue University (West Lafayette, IN), and 5085 of the resulting singe pass sequences were used for further analysis. Expressed sequence tags (ESTs) have been submitted to the NCBI dbEST (GenBank accession numbers BE640669–BE643506, BQ086920–BQ087668, and CV734654–CV736151).

EST Assembly

ESTs were filtered for short entries or low complexity sequences using SeqClean (http://www.tigr.org/tdb/tgi/software). The resulting sequences were assembled with the TGIR Gene Indices clustering tools (TGICL; Pertea et al., 2003). Briefly, TGICL clusters all of the ESTs using minimum overlap length (40 bp) and percentage identity (95%) criteria. The initial clusters were then sent to an assembly program (CAP3; Huang and Madan, 1999) that attempts to create one or more contigs from each cluster.

The resulting sequences in the dataset are termed tentative unique genes (TUGs) and consist of two types of sequences: contigs and

singletons. Contigs are two or more ESTs which are presumed to represent the same transcript, and singletons are ESTs without significant similarity to any other ESTs. The set of TUGs, composed of the sets of contigs and singletons, represents the unique genes found in the EST collection.

TUG Identification and Functional Analysis

The identities of the TUGS were determined using BLASTX (Altschul et al., 1997) against the *Arabidopsis* proteome (ATH1_pep_cm_20040228; http://www.arabidopsis.org). Functional and localization categories of the *Ceratopteris* TUGs were assigned using the TAIR Gene Ontology terms associated with the locus of the best *Arabidopsis* BLAST match (http://www.arabidopsis.org).

An estimate of the total number of unique genes expressed in *Ceratopteris* spores was made using a nonparametric estimator typically used for the estimation of population size or species richness in ecological studies (Burnham et al., 1979; Brose et al., 2003). This calculation makes use of the number of random samples in which each species appears. In our studies we varied the sample size as a single 384-well sequencing plate, half a plate (192-wells), and two plates (768-wells). For each TUG, the number of samples in which it occurred was tabulated from the number of plates containing its constituent ESTs. Given an expected

percent coverage of approximately 25 to 30%, the fourth-order jackknife estimator was used (Brose et al., 2003), and the final percent coverage of expressed genes was determined by dividing the number of TUGs by the estimated number of unique genes.

Arabidopsis Tissue-Specific Genes

EST collections from *Arabidopsis* seed, leaf, root, and shoot cDNA libraries were downloaded from The Institute for Genomic Research (TIGR; www.tigr.org). The ESTs were pooled together and analyzed as above for production of clusters and singletons. The resulting TUGs were identified by BLAST analysis against the *Arabidopsis* transcriptome (ATH1_cDNA_cm_20040228; http://www.arabidopsis.org). Only TUGs with transcript matches longer than 100 bp and greater than 97% identity were retained. A set of *Arabidopsis* genes expressed in pollen was also obtained from Honys and Twell (2003).

Reverse-Transcription PCR

Primers were generated based on sequence data available at NCBI for the following ESTs; BE641602, BE642746, BQ087334, BE641661, BE642715, BE642350, CV734685, and BE642120 (Table 2.4). One microgram total RNA from dry spores, 20 h spores, 14 day old gametophytes, and mature sporophytes was used for reverse transcription with oligo $d(T)_{22}$ primer and superscript II reverse transcriptase enzyme

(Invitrogen, Carlsbad, CA) following manufacturer's protocol. RNA was treated with Amplification Grade Deoxyribonuclease I (Invitrogen, Carlsbad, CA) following manufacturer's protocol just prior to reverse transcription reaction. 20 µL RT reactions were diluted 1:4 with nuclease free water, and 10µl of dilute RT was used as template in 25 µL PCR reactions with Taq PCR Master Mix (Qiagen, Valencia, CA).

Microarray Construction

Spotted cDNA microarrays were printed following protocols in DNA Microarrays (Childs et al., 2003). 3,840 cDNA clones corresponding to the EST library described in Stout et al. (2003) and deposited in dbEST (GenBank accession numbers BE640669-BE643506 and BQ086920-BQ087668) were amplified by PCR with SP6 and T7 vector specific primers in 96-well plate format. Amplified cDNA from 8 yeast clones with no significant sequence similarity to known *Ceratopteris richardii* genes were included in arrays to serve as non-specific binding controls. Sheared and un-sheared yeast genomic DNA and *Ceratopteris richardii* genomic DNA were also included as non-specific binding spots.

Poly-L-Lysine coated slides were produced following the protocol in DNA Microarrays (Childs et al., 2003) and available at www.microarrays.org. 32 tips in 4X8 configuration were used with Array Maker 2.4 software for array printing. Arrays were re-hydrated, blocked

and post-processed following the protocol found in DNA Microarrays (Childs et al., 2003) and used within three weeks of post-processing.

Fluorescent Probe Synthesis and Data Acquisition

Amino allyl dUTP Cy3 and Cy5 labeled microarray probes were synthesized following protocols adapted from DNA Microarray (DeRisi, 2003). Equal quantities of total RNA (15-30 μg) from two time points were used for each array probe. Reverse transcription with oligo $d(T)_{22}$ primer and superscript II reverse transcriptase enzyme (Invitrogen, Carlsbad, CA) with amino-allyl dUTP (Ambion, Austin, TX) were used to generate first strand cDNA. The cDNA sample from each time point was conjugated to one of Cy3 or Cy5 dyes (Amersham Biosciences, Buckinghamshire, England) and dye-swapped probes were included in this study. Two μl of 10 mg/mL sheared herring sperm DNA was included in each 50 μl probe for non-specific binding. The probe was applied to an array with 22X401 LifterSlip cover slides (Erie Scientific, Portsmouth, NH) and allowed to hybridize for ~6 h at 65° in humid array hybridization chambers (Corning, Corning, NY). Detailed descriptions of each array hybridization included in this study can be found in Supplemental Data.

Immediately following hybridization, the chambers were disassembled and arrays washed according to the method at http://chipmunk.icmb.utexas.edu/ilcrc/protocols/index.shtml. In some

cases Dye Saver 1 (Genisphere, Hatfield, PA) was used prior to scanning to prevent unequal degradation of the two dyes. Arrays were scanned following the protocol available at http://chipmunk.icmb.utexas.edu/ilcrc/protocols/Scanning.pdf using an Axon 4000 scanner (Molecular Devices, Union City, CA) and Axon GenePix Pro 4.1 or 5.1 software. The automatic flagging feature to identify "spots not found" was used. After manually griding arrays to correct for spot identification errors in the automatic spot location feature of genepix, array images, settings, and results files were uploaded into the Longhorn Array Database (Killion et al., 2003) and are available (Supplemental Data). In order to compare separate array hybridizations, normalization of arrays was calculated based on median log ratio equal to zero. The only R/G normalized mean values of spots that were retrieved and compiled for analysis using BAGEL software (Townsend and Hartl, 2002) were those that met the following criteria; spots must have been un-flagged by genepix, must have a minimum of 50 spot pixels, and must have at least 50% of green-or red-spot pixels greater then background intensity plus two standard deviations. Any spots not meeting these criteria were omitted from further analysis.

Quantitative Real-Time RT-PCR

Total RNA was isolated and handled as above, with the exception that DNase treatment of the RNA was carried out just prior to the reverse transcription step as opposed to treatment of the entire RNA sample directly after it was isolated. One μg RNA from 0 h, 6 h, 12 h, and 48 h time points was reverse transcribed according to the manufacturer's instructions with oligo $d(T)_{22}$ primer and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) to generate first strand cDNA.

Quantitative Real-Time RT-PCR was performed on six TUGs, and expression changes were compared to microarray analysis. Fluorescent primers were designed using web-based LUX™ designer software (http://www.invitrogen.com/content.cfm?pageid=3978#PrimerDesign) from Invitrogen based on the EST sequences of BE640734 (APT1), BQ086953 (alpha-tubulin), BE643392, BE642763, BE642932, BE642028, BQ087159 and BE642674. APT1 and alpha-tubulin were chosen as control genes because of their general use as such in other systems, their lack of significant expression changes in microarray analysis, and their minimal sequence similarity to other *Ceratopteris* ESTs. Control gene primers were labeled with JOE while all experimental genes were labeled with FAM and primer sequences are available (Table 2.3). PCR reactions were performed in 96-well polypropylene microplates using Platinum®

Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA) according to the Invitrogen cycling programs and protocols at one half final volumes (25 μl). A final concentration of 50 nM of appropriate gene-specific primers and the equivalent of 100 ng of reverse transcribed RNA were used per reaction. PCR and fluorescence measurements were carried out with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster, CA) as an absolute quantification run and initial data analysis was done with the manufacturer supplied SDS 2.2 software. Single target amplification of all samples and absence of genomic DNA in mock RT controls was verified via dissociation curve analysis. Fold expression changes were calculated using the comparative C_T method (User Bulletin #2: ABI Prism 7700 Sequence Detection System) in Microsoft Excel. For comparison to microarray BAGEL expression patterns, real time RT-PCR expression change values were normalized to the lowest expression value.

Acknowledgement of Assistance

All the microarray data were generated by the author (M. Salmi). The analyses of these data shown in Table 2.1 and Figures 2.1, 2.2, and 2.3 were carried out by computational methods, using public domain programs, by S. Stout with continuous input and discussion from the

author. The Real-Time PCR experiments were carried out jointly by the author and T. Bushart at the University of Texas Core Facilities.

RESULTS

Radiolabeled Nucleotide Incorporation Assay

The different concentrations of tritiated dUTP used showed the expected dose response curve of incorporation into spore RNA. The rate of tritium incorporation increased steadily over the first 24 h of development, with no significant increase in incorporation between 24 and 48 h. There was no effect on germination rate or morphology observed in spores treated with only tritiated dUTP. The concentration 0.6 μCi/mL was decided upon as the dose of tritiated dUTP to use in actinomycin D treatment experiments.

Inhibition of Transcription with Actinomycin D Treatment

The following concentrations of actinomycin D were tested on spores allowed to develop for 24 h before freezing; 0.2, 2, 6, 15, 20, and 40 μg/mL. Scintillation counts on 10 μg of RNA showed that after 24 h of treatment, 15 μg/mL of actinomycin D was sufficient to reduce the level of tritium detected in RNA samples to background levels (data not shown).

The percent of germinated spores was counted in actinomycin D treated and control samples at four different time points-after

illumination, 60, 72, 84 and 96 h (Fig. 2.1). 60 h after light exposure, 28% of control spores were germinated while only 9% of actinomycin D treated spores were germinated. After 72 h in light, almost 60% of control spores were germinated while only around 37% of actinomycin D treated spores germinated. By 84 h after light exposure, the germination rate of control and treated samples was equal at 68%. When spores were counted after 96 h in light, the control sample's germination rate (82%) was again higher than actinomycin D treated (70%). Compared to control, 50% less treated spores had prothallus development after 96 h. At all time points analyzed there was no difference in direction of primary rhizoid growth of treated and control spores.

Functional and Compartmental Categorization of the *Ceratopteris* TUGs

Clustering and assembly of the ESTs yielded 3,930 tentative unique genes (TUGs), composed of 513 contigs and 3,417 singletons. Contigs are consensus sequences generated from two or more ESTs that are determined to represent the same expressed gene, while singletons are ESTs with no strict similarity to other ESTs in the collection.

The estimate of the total number of genes being expressed in spores 20 h after light initiation varies slightly depending on the sample size used in the calculation. Over the range of sample sizes we used (a single 384-

well sequencing plate, half a plate (192-wells), or two plates (768-wells)), we estimate there to be between 14,317 and 15,297 unique genes expressed 20 h after light initiation of spore germination. This estimate indicates that the current 3,930 TUGs represent approximately 25.7 to 27.6% of those sequences.

The TUGs were identified by BLAST analysis against the *Arabidopsis* proteome, yielding 2,710 TUGs with significant similarity (E-value $\leq 1.0 \times 10^{-10}$) to *Arabidopsis* proteins. Using the Gene Ontology terms of the matching *Arabidopsis* loci (Berardini et al., 2004), functional and localization assignments were made for each of these *Ceratopteris* TUGs, and only genes with an assignable function or subcellular localization are presented. In order to determine what may represent typical functional and compartmental distributions, as well as provide a basis for comparison, genetic loci expressed in *Arabidopsis* seed, pollen, and leaf tissue were similarly analyzed.

The functional expression patterns seen in *Ceratopteris* spores were generally similar to those found in various *Arabidopsis* tissues previously sampled (Fig. 2.2). In each of the sets of loci, the broad categories of metabolism and protein metabolism were the most abundant, accounting for more than 45% of the genes with assignable functions (Fig. 2.2). The compartmental distribution of genes was more

variable between the *Ceratopteris* EST collection and the *Arabidopsis* tissues (Fig. 2.3). The largest difference was seen in the number of genes with their localization classified as "Other Membranes," where *Ceratopteris* spores had a proportion 5 to 10% smaller than those typically seen in *Arabidopsis*. The "Other Membranes" compartmental category is defined as membrane proteins, excluding those that localize to the plasma membrane. The only other difference between the *Ceratopteris* spores and the three *Arabidopsis* tissues was seen for genes associated with the ribosome, which occurred 1.5 to 4-fold more frequently in the spores than in the *Arabidopsis* tissues (Fig. 2.3).

Comparison of Genes Expressed in *Ceratopteris* Spores and *Arabidopsis* Pollen and Seeds

Ceratopteris spores share similar biological and physiological characteristics with *Arabidopsis* pollen and seeds. Therefore, we examined which genes were expressed in all three developmental stages of these plant model organisms. In order to limit the comparison to genes that show relatively specific patterns of expression rather than genes that are broadly expressed, we first screened each of these gene sets with a set of vegetatively expressed genes derived from analysis of over 32,000 ESTs from *Arabidopsis* roots, shoots, and leaves. Of the genes included in the seed, spore, or pollen sets, 50 to 60% are also expressed in vegetative

tissues (Fig. 2.4). The *Arabidopsis* genes exhibiting pollen- and seed-specific expression were then compared with the genes expressed in spores to determine which genes are shared. Nearly 9% of the genes expressed in seeds or pollen are also expressed in *Ceratopteris* spores (Fig. 2.4 and Table 2.1) and eight genes are expressed in all three tissues (Table 2.1).

Gene-specific primers for the eight genes common between *Ceratopteris* spores and *Arabidopsis* seed and pollen were used to evaluate their expression at other stages of the *Ceratopteris* life cycle. Total RNA was isolated from the following four stages of development: dry spores, imbibed spores 20 h after light exposure, 14 day old mature gametophytes, and mature sporophytes. Semi-quantitative and end-point RT-PCR showed that the messages for all of these genes have their highest level of abundance in 20 h developing spores, the time point used for this cDNA library. Messages for two genes, BE641602 putative Mago nashi, and BE642715 putative peroxisomal targeting signal type 1 receptor, were only detected in developing spores and not present in dry spores, mature gametophyte or mature sporophyte plants (data not shown).

Early Developmental Transcription Profile in *Ceratopteris* Spores

In order to analyze changes in gene expression during early spore development, four pair-wise time point comparisons were done with a

minimum of eight replications for each comparison; 0 h vs. 24 h, 6 h vs. 24h, 12 h vs. 24 h , and 48 h vs. 24 h. At least five different total RNA samples from each time point were used to generate probes for these comparisons. Complete data that adhere to MIAME standards (Brazma et al., 2001) for all arrays included in this study can be obtained (Supplemental Data).

Relative abundance of transcripts at 0 h, 6 h, 12 h, 24 h, and 48 h after light exposures was determined using BAGEL software (Townsend and Hartl, 2002). For statistical analysis by BAGEL software, an array spot must meet the quality criteria described in methods for a minimum of three array data sets for each of the four time point comparisons. Seventy-two spots were omitted from analysis because of this type of insufficient replication. Because of the comparative, quantitative nature of microarray analysis, BAGEL software presents the treatment (or developmental time point in this study) with the lowest expression level with the value one, and presents all other treatments as fold increases over one. This analysis also provides 95% credible interval range for all features at all treatment conditions (time points) included.

Changes in transcript abundance are defined as non-overlapping 95% credible interval between any two time points. Of the TUGs analyzed, 70% showed no significant change at any time point over the first 48 h of

development. 922 TUGs (29%) showed a significant difference between at least two of the developmental time points analyzed (Supplemental Data Table 2.5). Of TUGs that showed a change in transcript abundance, 138 (15%) were significantly more abundant 48 h after light exposure than upon germination initiation by light, or were up-regulated during the first 48 h of development. Included in that list are 35 TUGs that were significantly more abundant 48 h after light exposure than at all other developmental time points analyzed (Table 2.2). 203 TUGs (22%) were more abundant at the time of initial light exposure (0 h) than 48 h after light exposure, or they were down regulated over the first 48 h of development.

Many of the TUGs that show changes in expression over the first 48 h of development could be predicted to play key roles in directing early gametophytic development. Among these, we chose to compare and contrast the expression pattern of six TUGs that encode: two Mago nashi proteins, one SIN-like family member (Fig. 2.5A), a RAS-related GTP-binding protein, a member of the NPH3 family of proteins, and a catalase family member (Fig. 2.5B).

Two *Ceratopteris* TUGs that code for proteins with significant sequence similarity (E value of 1 X E^{-77}) to a previously described *Marsilea vestita* (fern) Mago nashi protein have differing expression patterns. The

mRNA for one *Ceratopteris* Mago nashi TUG has an increase in abundance that peaks 12 h after initial light exposure then steadily decreases through 48 h. Message for the second *Ceratopteris* Mago nashi TUG shows a steady increase in abundance throughout the first 48 h of development. Expression of the TUG for a protein with similarity (E value of 7×10^{-10}) to *Arabidopsis* SIN-like family protein parallels expression of one of the Mago nashi TUGs, showing peak expression at 12 h then steadily decreasing through 48 h.

The TUG encoding a protein with high sequence similarity (E value of 1×10^{-56}) to RAS-related GTP binding protein in *Arabidopsis*, shows steady expression throughout the first 24 h of development, with no significant difference in message abundance between any of these time points, followed by a doubling in abundance 48 h after initial light exposure. Abundance of the mRNA for a protein with high sequence similarity (E value of 3×10^{-23}) to the NPH3 family protein from *Arabidopsis* is 2-fold higher at the 0 h time point than 48 h after light exposure, with a steady decrease in abundance between the extreme points in development that were analyzed. Lastly, the TUG that encodes a protein with high sequence similarity (E value of 8×10^{-96}) to catalase chain 1 of upland cotton, *Gossypium hirsutum*, as well as strong similarity to

catalase genes from several other plants, is significantly up-regulated between 0 and 48 h after light exposure.

Verification of Microarray Patterns by Quantitative Real-Time RT PCR

As an independent confirmation of RNA expression patterns, Real Time RT-PCR was performed on six genes showing significant expression changes. Message levels for *Ceratopteris* sequences with the accession numbers BE642028, BE642763, BE642932, BE643392, BQ087159 and BE642674 were compared to those of two control genes, BQ086953 (alpha-tubulin) or BE640734, adenine phosphoribosyltransferase form 1 (APT1). RNA was isolated in the same manner as samples used for microarray experiments. Melting curve analysis showed discreet peaks for all samples, indicating amplification of single targets. Lack of detectable signals in mock RT controls demonstrated absence of genomic DNA contamination (data not shown). Expression pattern comparisons between microarray and real-time RT PCR are presented (Fig. 2.6, 2.7 and 2.8). General expression trends were corroborated by at least one control gene comparison. Specific fold changes were reasonably close to those estimated by the microarray results considering the longer linear range of sensitivity for Real Time RT-PCR.

DISCUSSION

Transcription is Necessary for Spore Germination

The finding of delayed germination rates in the absence of transcription, suggests that RNA synthesis is necessary for spore germination. The compound used in this experiment to inhibit transcription, actinomycin D is unstable in light. The reduced germination of treated samples observed at 60 and 72 h followed by equivalent germination rate after 84 h indicates that a significant amount actinomycin D may become degraded after 24 h, resulting in the 24 h delay observed. This is also supported by the decreased percent of treated spores with prothallus development at 96 h. This study did not address the question of whether or not the messages and/or proteins needed for gravity perception and response are present in dormant spores, but does show that germination of this fern spore requires new messages.

Comparison of Gene Expression in Spores, Seeds, and Pollen

While the number of ESTs analyzed in this project is similar in scale to that of other libraries of specific tissues and developmental stages (Fernandes et al., 2002; Sawbridge et al., 2003; Shoemaker et al., 2003), this library is unique, because its biological source is a single cell and represents a plant gametophytic generation. The first cell division in

Ceratopteris typically takes place 48 h after light initiation of germination, approximately 24 h after the gravity-directed downward migration of the spore nucleus. Because the cDNA used for EST sequencing is based on RNA collected 20 h after initiation of germination, this collection of ESTs represents *Ceratopteris* gene expression at a single-celled stage of development.

Spores at this stage of development are transitioning from a dormant, desiccated state to a metabolically active one, analogous to the process of seed germination in angiosperms. This similarity may be more than superficial as both processes appear to involve the relatively specific expression of similar genes, including those that code for proteins related to desiccation and dormancy, as well as several aspartic or cysteine proteases (Table 2.1). In developing seeds, storage proteins are processed into mature subunits by aspartic and cysteine proteases upon their transport to specialized protein storage vacuoles (PSV). Proteases are also involved in seed germination to initiate and complete metabolism of the storage proteins (Muntz, 1996; Gruis et al., 2002; Gruis et al., 2004). Because transport to the PSV is critical, the shared expression of a seed-specific vacuolar processing enzyme may be an indicator that a similar protein storage strategy operates in both seeds and spores. It has been hypothesized that seed plants could have co-opted genes used for spore

dormancy to develop post-embryonic seed dormancy (Banks, 1999). The common, tissue specific expression of genes in both *Ceratopteris* spores and *Arabidopsis* seeds supports this hypothesis.

Biologically, fern spores are part of the haploid gametophytic generation and are equivalent to angiosperm microspores and megaspores, which divide to produce mature pollen grains and embryo sacs, respectively. Using an oligonucleotide-array, Honys and Twell (2003) identified approximately 1,000 genes expressed in *Arabidopsis* pollen and estimated that the total number of pollen expressed genes is approximately 3,500. A similar number has also been predicted for *Arabidopsis* pollen using a Serial Analysis of Gene Expression approach (Lee and Lee, 2003). Estimates from our EST analysis place the number of genes expressed in germinating *Ceratopteris* spores at over 14,000. The substantial difference in estimated gene diversity between these two relatively simple germinating systems may reflect the relative physiological complexity of the free-living photosynthetic gametophytes in homosporous ferns as compared to the reduced, non-photosynthetic gametophytes of angiosperms. Along this line of reasoning, the two *Ceratopteris* TUGs that have the highest level of up-regulation between initial light exposure (0 h) and 48 h after light exposure both have high similarity to chloroplast hypothetical proteins. This gene is

uncharacterized beyond chloroplast targeting, and in Ceratopteris spores they are up-regulated nine to ten fold in the first 48 h of gametophyte development.

The unique group of genes found in *Arabidopsis* seeds, *Arabidopsis* pollen and *Ceratopteris* spores, but not included in cDNA libraries from *Arabidopsis* shoot, root or leaf (Fig. 2.4 and Table 2.1), provide good candidates for study of the physiological processes shared by these three stages; namely maintenance of and emergence from a desiccated, metabolically dormant state. Several of these genes have been previously implicated in processes related to germination, although the presence and functional roles of these genes in all three of these unique systems have not been commented on before.

The shared expression of a eukaryotic translation initiation factor, locus AT1G54290, in *Arabidopsis* seed, pollen and *Ceratopteris* spores comes as no surprise. It is well documented that inhibition of translation by treatment with the drug cycloheximide inhibits pollen germination (Metcalf et al., 2004; Fernando et al., 2001) as well as seed germination (Rajjou et al., 2004). We have also found that spores treated continuously with cycloheximide do not germinate (SC Stout and SJ Roux, data not shown).

These three systems are poised for extensive and rapid growth through cell division and/or cell expansion, therefore the inclusion of a subunit of the mitochondrial NADH-ubiquinone oxidoreductoase complex (Heazlewood et al., 2003), locus AT2G02050, in these three systems is also not surprising. In keeping with the theme of increased cellular metabolism, locus AT2G17370 encodes HMG-CoA reductase 2 (HMGR2), another gene found in all three systems. This enzyme catalyzes the synthesis of mevalonate, a precursor of plant isoprenoids, which are a diverse group of compounds including the hormones abscisic acid and gibberellins, sterols, components of the electron transport chain, and some plant defense agents (Enjuto et al., 1994).

The *Arabidopsis* locus AT2G25110 has been identified by full length cDNA sequence similarity as an MIR-domain containing protein, similar to stromal cell-derived factor 2 precursor (SDF-2) of *Homo sapiens*. The stromal cell-derived factor 2 is a secreted protein that acts as a chemoattractant in mammalian immune system cells (Hamada et al., 1996), and this report of its expression in *Arabidopsis* seed, pollen and *Ceratopteris* spore is, to our knowledge, the first indication of a potential role in plants.

Control of membrane trafficking is accomplished by SNAP- and SNARE-type targeting protein systems in most eukaryotes. These systems

have multiple small peptide components that provide specificity, and the *Arabidopsis* genome contains several of these genes, including 14 synaptobrevin, or VAMP, family proteins (Sanderfoot et al., 2000). Vesicular trafficking and vacuolar sorting are processes important in seed germination and pollen tube elongation, and we have identified one synaptobrevin family gene, locus AT2G32670, that has shared expression in seed and pollen and a likely homolog expressed in spores. This shared expression may help in identifying the specific roles of various SNAP/SNARE targeting components in plant growth and development.

The TUG found in *Ceratopteris* spores that is similar to the *Arabidopsis* gene AT4G25650 found in seed and pollen is described as rieske (2Fe-2S) domain-containing protein, similar to cell death suppressor protein lls1 from *Zea mays*. The gene, named lethal leaf spot1 (lls1) from *Zea mays,* was originally described as encoding a novel protein highly conserved in plants that functions as a cell death suppressor (Gray et al., 1997). Recently, it was determined that the Lls1 and its known orthologue in *Arabidopsis*, accelerated cell death 1 (Acd1) (Yang et al., 2004), are both genes that encode pheophorbide a oxygenase (PaO). PaO is an enzyme necessary for the catabolism of chlorophyll b and removal of a phototoxic intermediate in the pathway of chlorophyll degradation (Pruzinska et al., 2003). This gene is highly conserved among land plants,

and it has been found in several cyanobacteria (Gray et al., 2004), so the presence of a similar gene in *Ceratopteris* could be predicted. What may seem unlikely is the expression of a gene involved in chlorophyll catabolism in the non-photosynthetic spore, as well as pollen and seed of *Arabidopsis*. This is parallel to the finding of low level expression of Lls1 in non-photosynthetic maize embryos, endosperm, and roots, and has been explained by the postulate that all plant cells have an ability to degrade chlorophyll, regardless of the presence of chlorophyll in the cell (Yang et al., 2004).

The importance of the enzymes located in the peroxisome for lipid metabolism and reactive oxygen species scavenging has long been known (reviewed in Palma et al., 2002; Titorenko and Rachubinski, 2004). Recently, peroxisome-mediated signaling has been implicated in the nitric oxide signaling of stress responses (Murgia et al., 2004) as well as in the photomorphogenesis and development of seedlings (Hu et al., 2002). The *Arabidopsis* gene COMATOSE (CTS) is a homologue of the human adrenoleukodystrophy protein that is involved in transport of very long chain fatty acids into peroxisomes, and this gene has been implicated specifically in seed germination (Footitt et al., 2002). The presence of peroxisomal targeting signal type 1 (PTS1) receptor gene (PEX5) in seed, pollen and *Ceratopteris* spores provides evidence of the importance of

peroxisomal activity in the process of emerging from a desiccated dormant state that is common to all three (Table 2.1). Almost two hundred genes with PTS1 targeting signals for the peroxisome have been identified in the *Arabidopsis* proteome (Kamada et al., 2003). In tobacco, yeast, and humans PEX5 serves as part of the receptor of proteins bound for the peroxisome that contain the PTS1 motif (Kragler et al., 1998). Catalase is an enzyme found in the peroxisome that breaks down hydrogen peroxide very efficiently, and it may be targeted to the peroxisome via a PTS1 signal (Kamigaki et al., 2003). Further evidence for the importance of peroxisome activity in germinating systems is the significant up-regulation of a *Ceratopteris* catalase TUG (Fig. 2.5B) in the early development of germinating spores.

Analysis of Early Development Expression Profiles

The majority of TUGs analyzed, around 70%, show no credible change between any two time points of development analyzed. The remaining 922 TUGs that show a significant change between at least two time points (Supplemental Data Table 2.5) were analyzed for trends in expression pattern relevant to the physiological processes of developing spores.

Around 48 h after light exposure, the single cell of the spore undergoes its first division. The 138 TUGs that are up regulated between

initial light exposure and 48 h later may be involved in the spore emerging from its dormant state, or preparing for and undergoing this cell division. There is a subset of this group of TUGs that shows a unique expression pattern: 35 TUGs (Table 2.2) show no change in transcript abundance over the first 24 h of development, but the expression level 48 h after light exposure is significantly higher than at all other time points. This expression pattern suggests that these genes may be needed for spore cell division.

One TUG which shows this pattern of change in abundance has strong similarity (E value of 1×10^{-56}) to *Arabidopsis* Ras-related GTP-binding protein (Fig. 2.5B). *Arabidopsis* Gα subunit (GPA1) and G protein coupled receptor (GCR1) play a role in the cell proliferation that occurs during seed germination (Jones and Assmann, 2004). The role of G protein signaling in plant cell division and proliferation may not be restricted to angiosperms, but may be a fundamental signaling pathway common to all plants as suggested by this expression pattern.

Another interesting pattern of expression includes genes which are possibly involved in maintaining and breaking the dormancy of the spore; 203 TUGs which are significantly more abundant when the spores are first exposed to light (0 h) than 48 h later. This is almost twice the number of TUGs which are significantly up-regulated over the first 48 h of

development. The emergence from dormancy of fern spores is a light activated, phytochrome mediated response (Cooke et al., 1987). One TUG with this expression pattern has strong sequence similarity (E value of 3 X 10^{-23}) to the NPH3 family protein from *Arabidopsis thaliana* that is critical for phototropism (Fig. 2.5B). Several other TUGs with this expression pattern show strong similarity to signal transduction pathway elements and should be studied for their roles in the process of breaking dormancy and germination.

RNA Localization in Spore Polarity Development

Ceratopteris spores determine the polarity of their subsequent development as a response to the vector of gravity some time between 6 and 18 h after they are initially exposed to light. For this reason genes that undergo changes in transcript abundance during this period of development are of particular interest. The mechanisms by which plant cells determine developmental polarity and cell fate are only beginning to be unraveled (Vroemen et al., 1999; Grebe et al., 2001; Cove, 2000).

The *Drosophila* oocyte is a model system for studying the establishment of cell polarity with well-characterized molecular components. Among these molecular components is a protein called Mago nashi which appears to be highly conserved across kingdoms (Swidzinski et al., 2001). In *Drosophila* and *C. elegans,* Mago nashi family proteins

function as structural components of the splicesome that are important in oogenesis. *Drosophila* Mago nashi protein is necessary for proper localization of at least one other mRNA, Oskar, and the proper localization of this mRNA during polar development is required for normal oocyte development (Hatchet and Ephrussi, 2004; Micklem et al., 1997). An *Arabidopsis* analog of Mago nashi is found in both seed and pollen (Table 2.1), and two distinct *Ceratopteris* TUGs with significant (1 X E^{-77}) similarity to a previously described Mago nashi protein in the fern *Marsilea vestita* were included in this EST library. One of the Mago nashi TUGs in developing spores (Fig. 2.5A) is up-regulated early in development, with its peak transcript abundance 12 h after light exposure, followed by steadily decreasing transcript levels at 24 h and 48 h. The other *Ceratopteris* Mago nashi TUG shows a steady increase in transcript abundance over the first 48 h of development, supporting our distinction of this as a separate *Ceratopteris* gene, and indicating that these two structurally similar proteins may be involved in different developmental processes (Fig. 2.5A). A role for RNA localization during plant embryogenesis was recently reviewed by Okita and Choi (2002).

The proposed *Arabidopsis* Dicer homolog, SIN1/SUS1/CAF, which is essential for embryogenesis (Golden et al., 2002) demonstrates another important role for post transcriptional modifications in the early

development of plants. A *Ceratopteris* TUG that is significantly up-regulated during the period of polarity determination in spores shows significant sequence similarity (E value of 7 X 10^{-10}) to an *Arabidopsis* SIN-like family protein (Fig. 2.5A). The involvement of SIN-like and Mago nashi-like genes in animal oocyte polar development, the specific expression of Mago nashi RNA in *Arabidopsis* seed and pollen, and the pattern of Mago nashi- and SIN-like transcript changes in spore germination suggests a role for RNA splicing and localization in polar development of cells in plants as well as animals.

Table 2.1 Genes expressed in *Ceratopteris* spore and/or *Arabidopsis* pollen and seeds. Genes were selected on the basis of abundance in the respective tissues.

Expressed in Arabidopsis seed, Arabidopsis pollen, and Ceratopteris spore

AGI Number	Protein Description	No. of ESTs (seed/spore)
AT1G02140	Mago nashi family protein	2/2
AT1G54290	Eukaryotic translation initiation factor SUI1	1/1
AT2G02050	NADH-ubiquinone oxidoreductase B18 subunit	1/1
AT2G17370	HMG-CoA reductase 2 (HMGR2)	3/1
AT2G25110	MIR domain-containing protein	2/1
AT2G32670	Synaptobrevin family protein (VAMP)	1/1
AT4G25650	Similar to cell death suppressor protein	4/2
AT5G56290	Peroxisomal targeting signal type 1 (PEX5)	2/1

Expressed in Arabidopsis seed and Ceratopteris spore

AGI Number	Protein Description	No. of ESTs (seed/spore)
AT3G62730	Desiccation related protein	56/1
AT1G62290	Aspartyl protease family protein	33/2
AT1G54870	Short-chain dehydorgenase/reductase (SDR) family protein, C-terminal similar to dormancy related protein	13/4
AT1G62710	Vacuolar processing enzyme specific to seeds	9/2
AT3G54940	Cysteine proteinase	5/7
AT5G50260	Cysteine proteinase	4/11
AT3G15670	Late embryogenesis abundant protein	2/10
AT5G03860	Malate synthase, strong similarity to glyoxysomal malate synthase	1/8

Expressed in Arabidopsis pollen and Ceratopteris spore

AGI Number	Protein Description	No. of ESTs (spore)[1]
AT2G21870	Expressed protein	4
AT2G32910	Expressed protein	4
AT5G20620	Ubiquitin polyprotein	4
AT1G04290	Thioesterase family protein	3
AT1G13950	Eukaryotic translation initiation factor 5A-1	3
AT1G23750	DNA-binding protein-related protein	3
AT3G51840	Short-chain acyl-CoA oxidase	2
AT1G51260	Acyl-CoA:1-acylglycerol-3-phosphate acyltransferase	1

[1] Pollen expressed genes were obtained from oligo-array data, so no EST abundance data are available (Honys and Twell, 2003).

Table 2.2 *Ceratopteris* TUGs up regulated at 48h. Transcripts that are significantly more abundant 48 h after light exposure then at all other time points analyzed. There are no significant differences between 0 h and 24 h time points. BLAST match identities based on NCBI non-redudant BLASTX with E value of less then $1E^{-10}$.

***Ceratopteris* Accession**	**Significant BLASTX match**	**48 h Mean fold increase**
BE642932	Ras-related GTP-binding protein, putative	1.84614
BE642821	No Significant Match	1.86453
BE640953	No Significant Match	1.88939
BE641666	RNase L inhibitor-like protein	1.91909
BE643423	No Significant Match	2.09459
BE642170	Caltractin (Centrin)	2.19707
BE643189	Actin	2.224
BE642902	No Significant Match	2.30533
BE642238	hypothetical protein	2.34746
BE641883	60S ribosomal protein L30	2.36364
BE640681	Luminal binding protein 4 precursor	2.38416
BE643260	Aspartate transaminase AAT1	2.41254
BE641191	ADP-ribosylation factor-like protein	2.42771
BE642609	expressed protein	2.43561
BE642799	Tubulin alpha-2 chain	2.48786
BQ087131	No Significant Match	2.50474
BE641972	No Significant Match	2.63733
BE642063	No Significant Match	2.64815
BE641956	Malate synthase	2.65116
BE642874	Actin	2.67442
BE641578	Citrate (si)-synthase	2.67893
BE641651	S-adenosyl-L-homocysteine hydrolase	2.79619
BE642201	DNA binding protein ACBF	2.86269
BE641696	No Significant Match	2.92608
BE640965	40S ribosomal protein S11 (RPS11B)	2.94158
BE641296	Isocitrate dehydrogenase [NADP]	3.03089
BE642895	S-adenosyl-L-methionine synthetase	3.24748
BE642724	S-adenosyl-L-homocysteine hydrolase	3.47217
BE642837	S-adenosyl-L-homocysteine hydrolase	4.13487
BE642043	No Significant Match	5.47248
BE642199	No Significant Match	5.73973
BE641757	No Significant Match	7.43751
BE641982	No Significant Match	9.26326
BE641063	Chloroplast hypothetical protein	9.49929
BE643187	Chloroplast hypothetical protein	9.64878

Table 2.3 Real-time RT PCR primers.

Target	Labeled forward primer	Unlabeled reverse primer
BE642763	GAACGGGATACATGGAAAGCGTTCGT[FAM]TC	AGGCTTGAAAGCATTTGAGGT
BE642028	CACACCTTTGTCCAGCGATTGGTG[FAM]G	CCAGCCTTGTGCCTCTGTGT
BE642932	GACTAAACACGCGCAACGAATTTAG[FAM]C	TCCCAGATTTGAGCCTTGATG
BE643392	CACGCGACTACATGATGATGCTGCG[FAM]G	CTCCCAAATTCTCAGGTTCCA
BQ087159	CACTTTGCGAATGCCCCTAAAG[FAM]G	CCAAATCACTGTCTATGCCAAT
BE642674	GAACGCGGGAGTCCAGGCCG[FAM]TC	TCTCCATGACAAGACCCTTCCA
BE640734	CACGGGTGCAAGAGGGTGACCG[JOE]G	CCACCTCTGCTCCAGCTCTC
BQ086953	CACTTTACTGGTGGTGATCTGGCTAAAG[JOE]G	ACACCTCGCAACACTTGTGGA

Table 2.4 Unlabeled RT PCR primers.

Ceratopteris target	Forward primer	Reverse primer
BE641602	TCGCTGATAGCGAGATAATGAA	GTGCAAACCAATAAGGGAAAAG
BE642746	CGTCTCGTTGAAACAGAAGTTG	TTCATGATAAGGATGAACAGCA
BQ087334	GTTTGCACGCATCTTATGTACC	TACTCTTTTCCGTCAACAAGCA
BE641661	CTCTCTCTCTTCCCGTCTGTGT	ATCTTTCTTCGAAGTTGCTTGC
BE642715	GAAGGGGAGACATGATAGATGC	TGAAACAAACAATGGCAAGTTC
BE642350	AAGTTGATATGGGCTTTCAGGA	GACAACCATCTTGAAGCAACAG
CV734685	ATGAGATTCCGTATGGATCAGG	ACGATCGTCACAGCTCACTCTA
BE642120	CCCGGTCAAGTTAAGGTTAATG	GACCAAAATGCAGCAGTAAACA

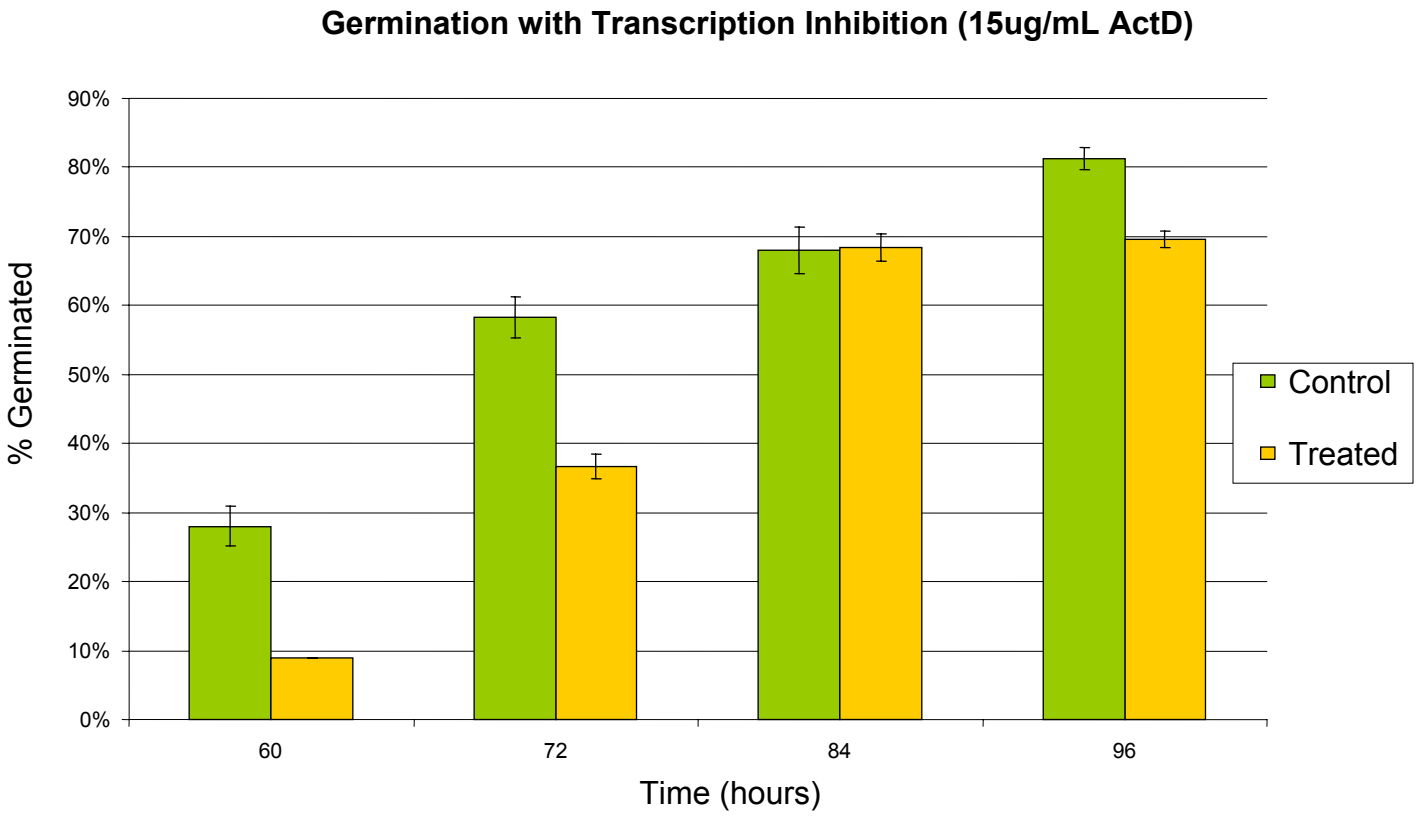


Figure 2.1 Germination in the presence of actinomycin D. Germination is considered the presence of the primary rhizoid. Approximately 100 spores were counted on each of three slides, except treated sample at 60 h, for which only two slides were counted. Error bars represent standard error

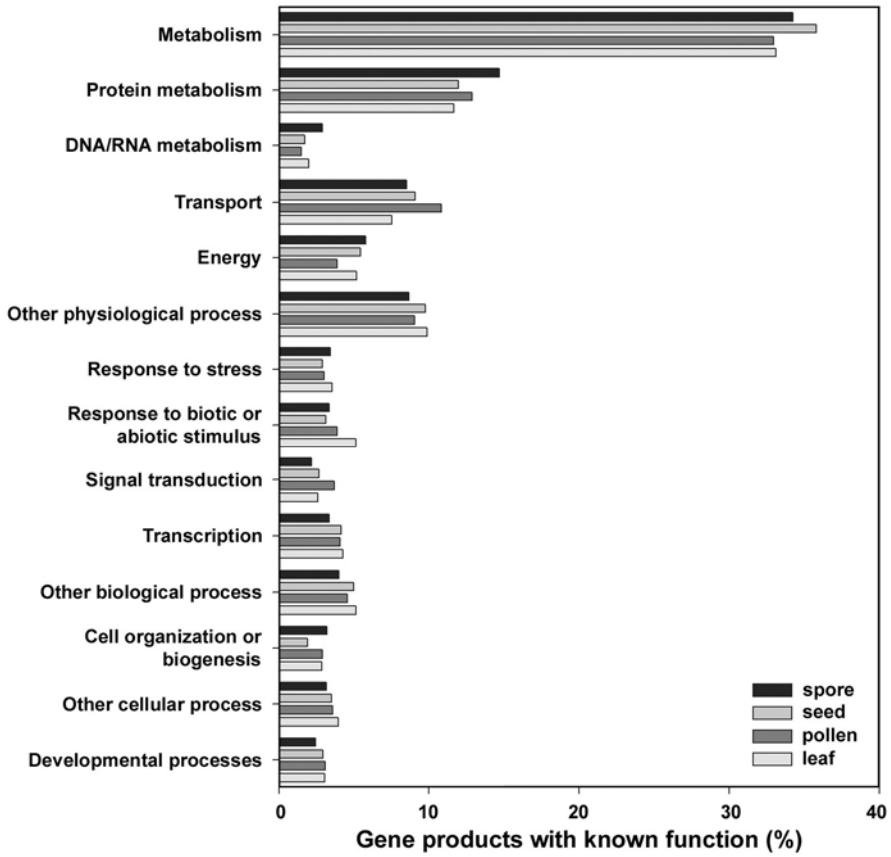


Figure 2.2 Functional classification of gene products expressed in *Ceratopteris* spores. *Ceratopteris* TUGs were annotated by BLAST comparison with the *Arabidopsis* proteome and the functional classification of each TUG was done according to the TAIR Gene Ontology database of the resulting best BLAST match. The functional classification distribution of genes expressed in *Arabidopsis* seed, pollen, and leaf tissue is also indicated.

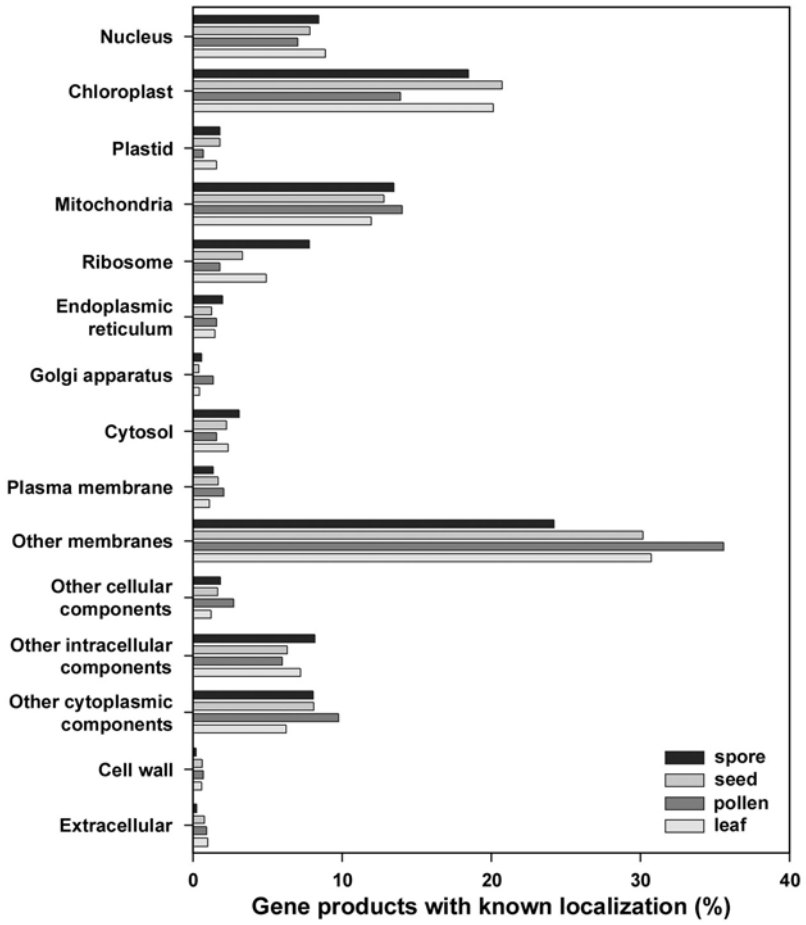


Figure 2.3 Localization of gene products expressed in *Ceratopteris* spores. *Ceratopteris* TUGs were annotated by BLAST comparison with the *Arabidopsis* proteome and the compartmental classification of each TUG was done according to the TAIR Gene Ontology database of the resulting best BLAST match. The compartmental distribution of genes expressed in *Arabidopsis* seed, pollen, and leaf tissue is also indicated.

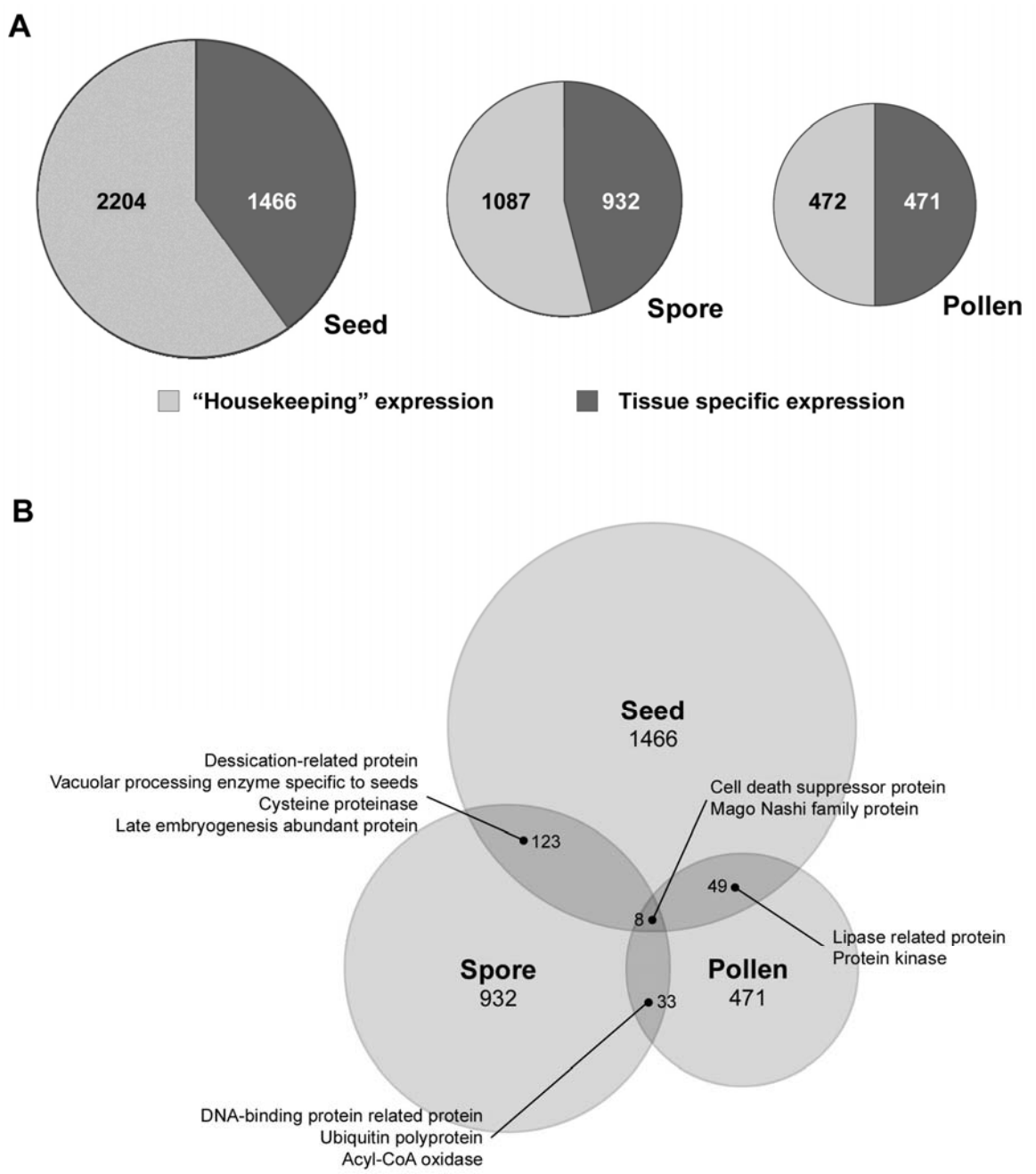


Figure 2.4 Comparison of the *Ceratopteris* spore TUGs to genes expressed in *Arabidopsis* seeds and pollen. (A) Identification of tissue-specific gene expression in *Ceratopteris* spores and *Arabidopsis* seeds and pollen. The proportion of genes present in the seed, spore, and pollen sets that were also expressed in vegetative tissue is indicated in light gray. The remaining tissue-specific genes, indicated in dark gray, are used for the expression comparisons. Numbers indicate the number of genes included in each proportion. (B) Overlapping expression of genes expressed in seeds, spores, and pollen. Numbers in overlapping regions indicate number of genes shared between the respective sets. Several relatively abundant genes present in each overlap are also indicated.

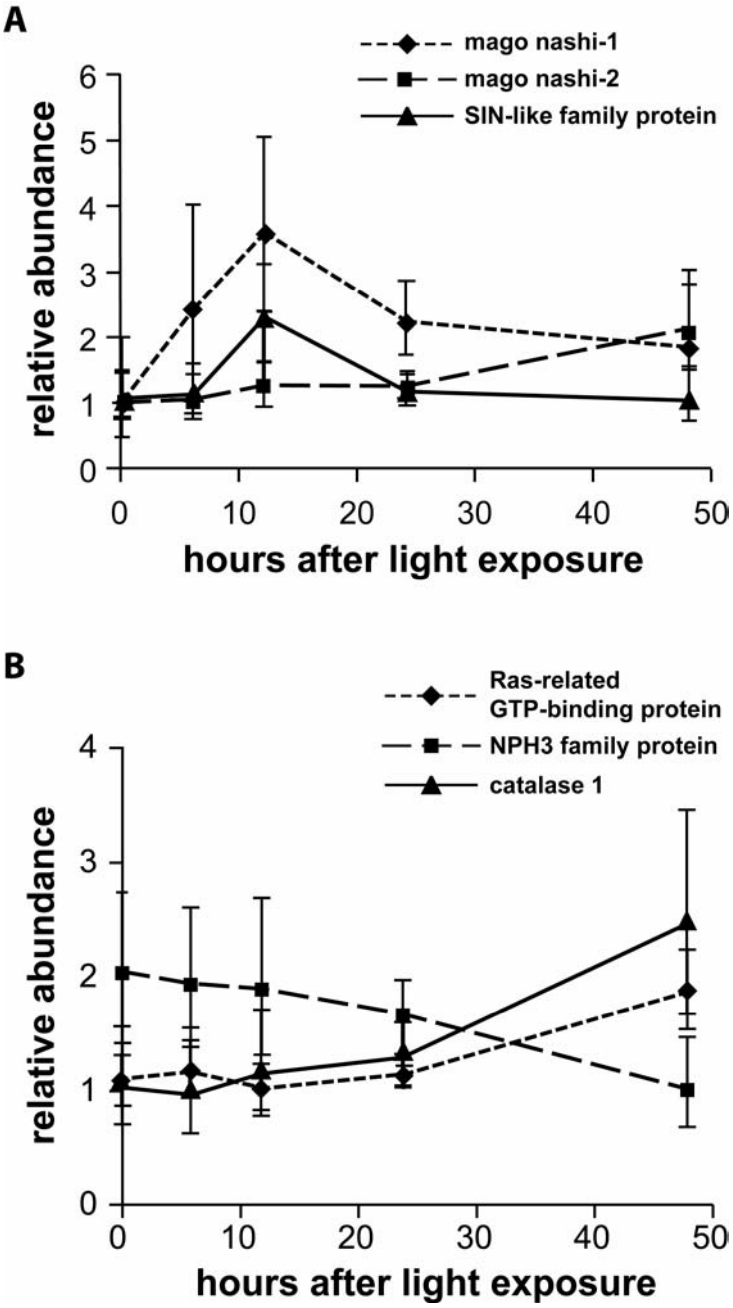


Figure 2.5 Specific expression patterns of *Ceratopteris* TUGs. Sequences are identified by accession number. The identity of the best BLASTX match to the *Ceratopteris* sequence is included. Times after initial light exposure tested were 0 h, 6 h, 12 h, 24 h, and 48 h. Transcript abundance at the time point with the lowest mean expression level is presented as 1 and all other time points are relative to 1. Error bars represent 95% credible interval for relative expression levels. Significant difference in RNA abundance between time points is defined as non-overlapping 95% credible interval. *Ceratopteris* accession numbers are as follows; (A) Mago nashi 1 = BE641602, Mago nashi-2 = BE642256, and SIN-like family protein = BE643380 (B) Ras-related GTP-binding protein = BE642932, NPH3 family protein = BE641485, and catalase 1 = BE642028.

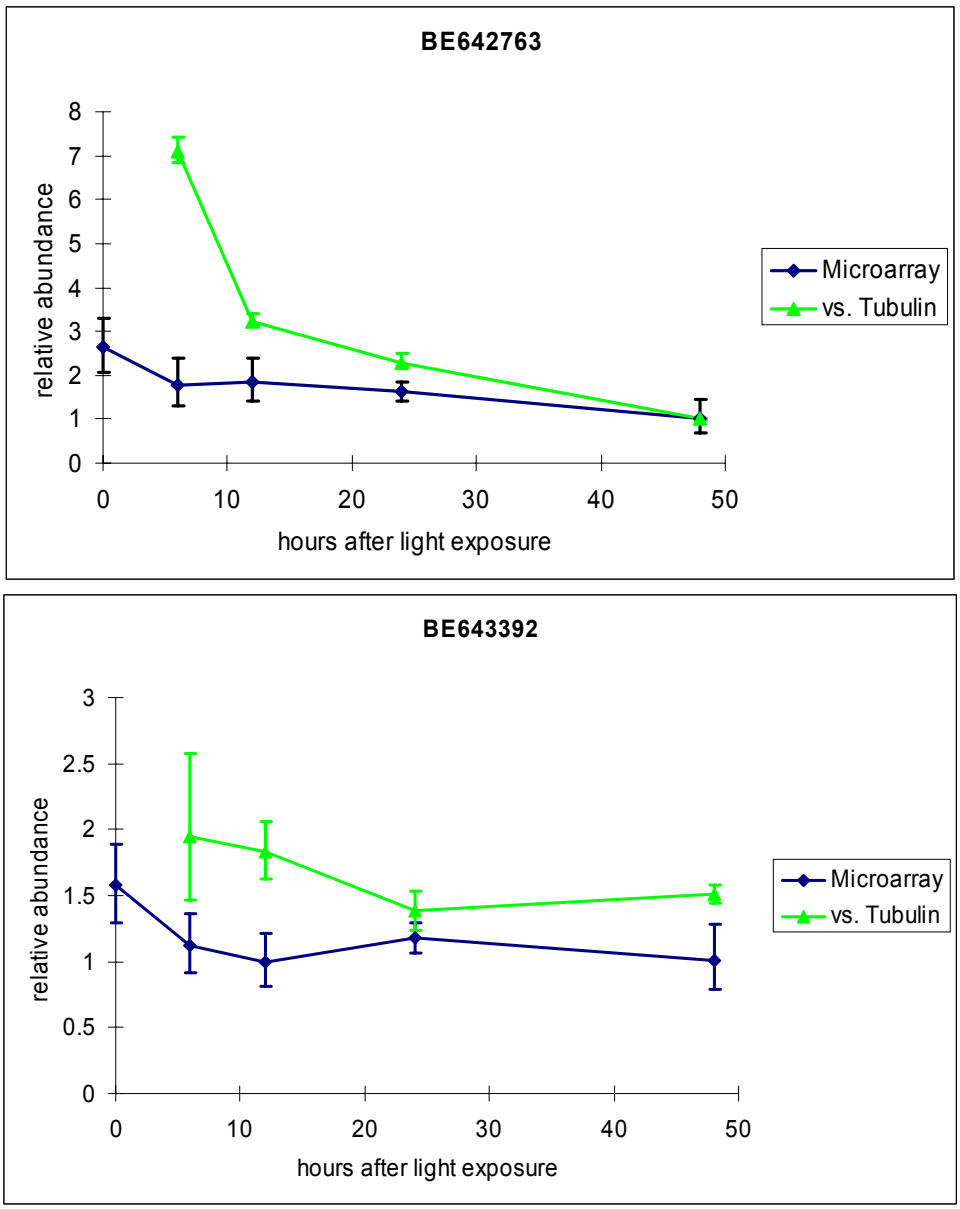


Figure 2.6 Real Time RT-PCR verification of microarray expression. Normalized expression levels are shown for genes in relation to control α-tubulin. 0 h time points for comparisons to control gene has been omitted due to consistently high expression readings at that time point regardless of the gene being tested, indicating that it is unsuitable as a control gene for this early time point. Error bars for Real Time RT-PCR represent a gene expression range based on standard deviation of three reverse transcription reactions. Microarray error bars are presented as 95% credible intervals. Ceratopteris genes are identified by accession number at the top of the graph.

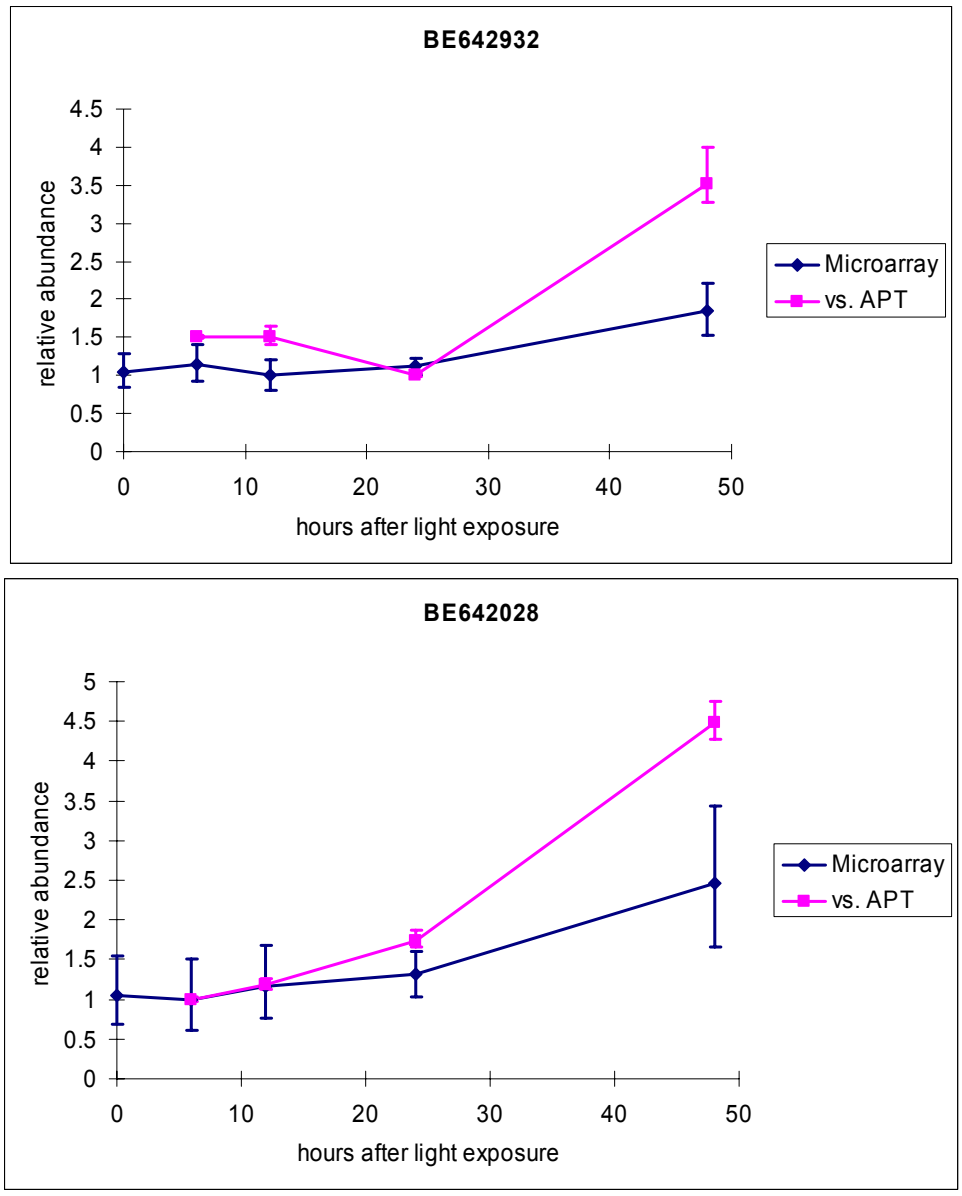


Figure 2.7 Real Time RT-PCR verification of microarray expression. Normalized expression levels are shown for genes in relation to control APT. 0 h time points for comparisons to control gene has been omitted due to consistently high expression readings at that time point regardless of the gene being tested, indicating that it is unsuitable as a control gene for this early time point. Error bars for Real Time RT-PCR represent a gene expression range based on standard deviation of three reverse transcription reactions. Microarray error bars are presented as 95% credible intervals. Ceratopteris genes are identified by accession number at the top of the graph.

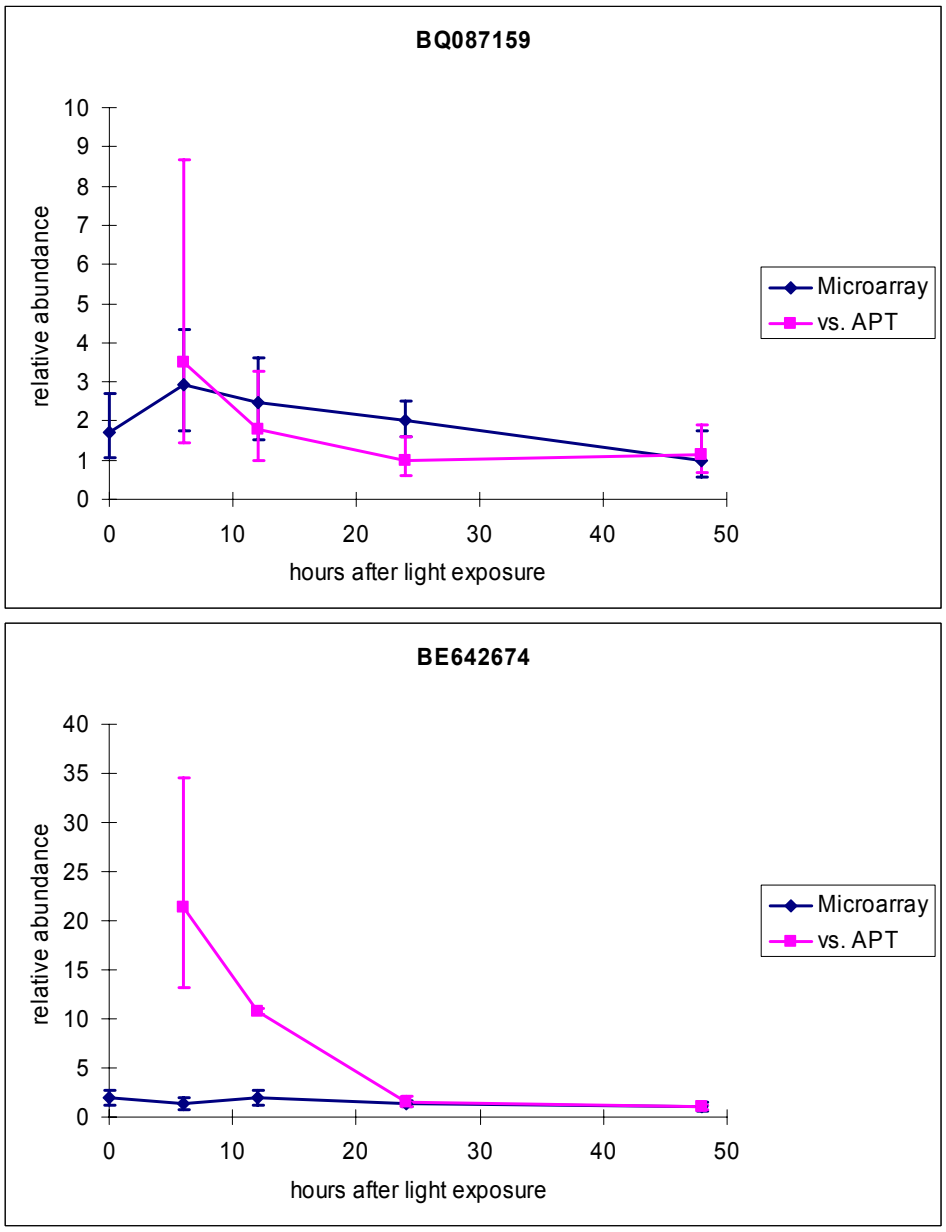


Figure 2.8 Real Time RT-PCR verification of microarray expression. Normalized expression levels are shown for genes in relation to control APT. 0 h time points for comparisons to control gene has been omitted due to consistently high expression readings at that time point regardless of the gene being tested, indicating that it is unsuitable as a control gene for this early time point. Error bars for Real Time RT-PCR represent a gene expression range based on standard deviation of three reverse transcription reactions. Microarray error bars are presented as 95% credible intervals. Ceratopteris genes are identified by accession number at the top of the graph.

CHAPTER 3

GENE EXPRESSION CHANGES THAT OCCUR IN GERMINATING SPORES IN REDUCED GRAVITY DURING SPACEFLIGHT

INTRODUCTION

Ceratopteris spores are a valuable system for studying the cellular components of gravity perception and the earliest steps of gravity-induced signal transduction. Gametophytes respond to the force of gravity during a limited window of time in their early development after emerging from dormancy (Edwards and Roux, 1998). The developmental timing of a population of spores can be synchronized by soaking spores in water, or aqueous media, in complete darkness for 4 days or more prior to exposure to light. Red light initiates the germination of imbibed spores. In a synchronous population 20 h after light exposure, 50% of spores have determined the directionality of their development as a response to gravity.

Ca^{2+} signaling has been implicated in this gravity-directed polarization of spores (Chatterjee et al., 2000). Within an hour after their germination is initiated by light, spores exhibit a trans-cell calcium current revealed by a calcium efflux from the top of germinating spores and influx

at the bottom of spores. Although the intracellular distribution of calcium ions has not been tested in spores, the calcium current detected outside the spore can re-orient as rapidly as 45 seconds after spores are rotated 180 degrees. This is the shortest time that the detection equipment is capable of measuring the re-orientation, so the current probably re-orients faster.

The gravity vector determines the direction that the cell nucleus will migrate around 24 h after light exposure, which is 20–30 h prior to the first cell division. In over 90% of spores, the nucleus migrates down and sets up an asymmetric cell division that results in a smaller upper cell that will develop into the gametophyte prothallus, and a larger lower cell that will develop into the primary rhizoid of the gametophyte. The primary rhizoid emerges from the broken spore coat 72–96 h after light exposure and grows down. Spores flown on NASA shuttle flight STS-93 definitively demonstrated that these polar events in the early development of the gametophyte are a response to gravity. Roux et al. (2003) observed that spores develop as described on earth, but the direction of nuclear migration and primary rhizoid growth in spores germinating in microgravity on the shuttle is random.

There are currently two differing models to explain the mechanism by which plant cells sense the directional force of gravity: the starch-

statolith sedimentation model and the gravitational pressure model. Evidence supporting the statolith sedimentation model was recently reviewed by Morita and Tasaka (2004). Briefly, statoliths, which occur in specific gravity sensing cells called statocytes, are typically starch-filled plastids that are more dense than their surrounding cytoplasm. In *Arabidopsis,* statocytes have been observed in the columella cells of the root cap and in a shoot cell layer between the stele and cortex (incorrectly referred to as the "endodermis"). Statoliths fall down within their cells when shoots or roots are reoriented. In the statolith sedimentation model it is the direction that these statoliths fall that serves as the signal to the cell of the directionality of the force of gravity. This model relies on an as yet unidentified intracellular receptor that detects the location of the statolith. In *Ceratopteris richardii* spores the pro-plastids encircle the nucleus and appear to move in concert with the nucleus. The nucleus and associated organelles move in random directions within oriented spores until after gravity has fixed the polarity of the cells (Edwards and Roux, 1998), making it unlikely that they could inform the cell about the direction of the gravity vector.

The gravitational pressure model, described by Staves (1997), suggests that it is the mass of the entire cell protoplasm that is pulled downward by gravity. This exerts pressure on the lower part of the cell wall

or extra cellular matrix and tension at the top of the cell, and these tension and compression forces are sensed by the cell through an unknown receptor that spans the plasma membrane and contacts the cell wall. In the gravitational pressure model, the statolith structure of a cell may be involved in gravity perception by contributing to the mass of the cell protoplasm. No cellular receptor mechanism for perception of gravity has been identified in any plant system. These differing models may both exist in different plant systems, or some variation of these models may prove to be the cellular gravity detection mechanism.

Most of the current literature that attempts a molecular characterization of plant gravity response focuses on the intercellular communication of the gravity signal and on how this directs organ bending (reviewed by Friml and Palme, 2002). Beyond the two hypotheses described above, little is known about the specific cellular mechanism for plant cell perception of gravity or the earliest intracellular events involved in transducing that signal into a cellular response.

The literature on signaling steps that help transduce the force of gravity into altered growth in plants is diverse and implicates numerous molecular participants. The role of Ca^{2+} signaling in gravity responses has been demonstrated by observations in many different plants, both on earth and in altered gravity conditions, as reviewed by Kordyum (2003).

Cytological observations suggest that the actin network of the cytoskeleton is involved in detection of amyloplasts sedimentation (Blancaflor, 2002). Fischer et al. (2004) review the involvement of lipid signaling and altered lipid composition in mediating gravity responses in plants. Rapid changes in cytosolic pH associated with *Arabidopsis* root gravity stimulation and gravitropism (Scott and Allen, 1999; Fasano et al., 2001) suggest that changes in cytosolic [H^+] may be one of earliest intracellular responses to gravity.

Comparing the development of cells in altered gravity conditions to that in normal earth gravity is an excellent approach to identifying the early cellular and molecular mechanisms of gravity signal perception and response. *Ceratopteris richardii* spores were flown on NASA shuttle flight STS-93 to allow this kind of comparison. The spores were kept in darkness, and therefore dormant, until the shuttle reached earth orbit, where the force of gravity drops to below .001 *g*. Once in orbit, spores were exposed to light sufficient to induce germination, and allowed to develop in microgravity before being frozen at various time points during the period when, on earth, gravity would direct their polarization. Frozen spores were returned to earth, and stored frozen until adequate methods to globally assess their gene expression profile could be developed.

The goal of the experiments described in this chapter was to document the difference in mRNA abundance induced spaceflight. Space-flown biological material is a limited and virtually irreplaceable resource, therefore microarray analysis was chosen as the most efficient way to monitor expression changes of a large number of genes. The expression differences of several genes revealed by microarray analysis have been verified by quantitative real-time RT-PCR. Stringent criteria and statistical analyses of replicated array comparisons have revealed the identity of *Ceratopteris* genes that are up-regulated by the spaceflight environment and others that are down-regulated by it. The genes in *Ceratopteris* whose expression differs between space and ground have been compared to those documented in other studies of changes induced by spaceflight in various plants and animals. We have found evidence that some of the biochemical processes implicated in gravity perception and early signal transduction in other plant systems may be part of the *Ceratopteris* gametophyte gravity response.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

Ceratopteris richardii spores of an inbred diploid strain designated Hn-n flown on NASA shuttle mission STS-93 are described in Roux et al. (2003). Spores were allowed to soak in sterile water in complete darkness

at 29° for 7 days prior to flight. One day prior to flight the water was removed, and spores were sown on Petri dishes in a solution of half strength Murashige and Skoog Basal Medium (Sigma-Aldrich, St. Louis, MO) 0.5% agarose at a concentration of 1 g in 10 ml. Petri dishes were individually sealed with parafilm and placed inside BRIC flight canisters and loaded on the shuttle. Spores remained dormant in darkness inside BRIC canisters until the second day in orbit, at which time spores were exposed to ambient light in the mid-deck for varying time points: 1 h, 8 h, and 20 h used in this study. After light exposure, spores were frozen in a gaseous nitrogen freezer and were kept frozen until total RNA was isolated. Ground-control spores were treated in the same manner as flight samples. Six plates were allowed to continue development onboard the shuttle to verify germination and growth of spores in microgravity. After the Shuttle returned to earth, these plates were compared to earth samples and no obvious difference in developmental stage or percent germination was observed (Roux personal communication).

RNA Isolation

Total RNA was isolated from frozen plates of spores in agar as described (Chapter 2). The higher density of spores used in the shuttle experiment resulted in a higher concentration of contaminating organic compounds (carbohydrates, phenols, peptides) in the RNA samples, giving

the samples a low (0.9–1.4) OD 260/230. To remove contamination from these samples the MEGAclear RNA purification kit (Ambion, Austin, TX) was used. RNA was eluted from the kit in 100 µL followed by ETOH precipitation. RNA was resuspended in 20 µL DEPC treated TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA) and OD 260/230 of purified RNA samples was above 2.1.

Second Microarray Construction

After construction of the microarray described in Chapter 2, an additional four 384 well plates of ESTs was obtained and deposited in dbEST (GenBank accession numbers CV734654 through CV736151). In order to have an equal amount of previously printed and new cDNA for these microarrays, PCR amplification of cDNA from all 14 plates was done in the same manner described (Chapter 2). Microarray construction with these amplified cDNAs, including the same non-specific binding controls, was done in the manner described in Chapter 2. Gene identity and annotation of *Ceratopteris* sequences used for this analysis and included on this microarray are described in Chapter 2.

Fluorescent Probe Synthesis and Data Acquisition

A direct incorporation method of fluorescent probe synthesis was used in this study, which eliminates the potential for differential degradation of red and green dyes. Reverse transcription with 5 µg oligo

$d(T)_{22}$ primer, 5 μg $pd(N)_6$ random hexamer (Amersham Biosciences, Buckinghamshire, England) and 2 μl superscript II reverse transcriptase enzyme (Invitrogen, Carlsbad, CA) was used to generate first strand cDNA. Unlabeled dNTP mix with 25 mM dATP, dCTP, and dGTP, and 15 mM dTTP was used in addition to 1 μl CyDye™3-dUTP or CyDye™5-dUTP (Amersham Biosciences, Buckinghamshire, England). After first strand cDNA synthesis, RNA was degraded by addition of 0.045 M NaOH and 70°C incubation for 10 minutes. Reactions were neutralized by the addition of 0.045 M HCl and cleaned using MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA).

Equal quantities of total RNA (10–20 μg) from one flight sample and the same time-point ground sample were used for each array probe. Two array hybridizations in each dye color configuration (flight=red and ground=green or the inverse) are included in this study; therefore, there are 4 replications of flight versus ground comparison at each of the following durations of light exposure: 1 h, 8 h, and 20 h. Array hybridization conditions, array washing, array scanning and data acquisition, and spot quality criteria were all done following methods described in Chapter 2.

Quantitative Real-Time RT-PCR

One μg of total RNA isolated for microarray hybridization described previously was used for quantitative real-time RT-PCR by the same methods described in Chapter 2. Quantitative Real-Time RT-PCR was performed on seven TUGs that had shown significant differences between flight and ground samples in microarray analysis, and expression changes were compared to those found by microarray analysis. LUX™ fluorescent primers were designed using Invitrogen's LUX™ Designer software (http://www.invitrogen.com/content.cfm?pageid=3978#PrimerDesign). The same control genes, BE640734 (APT1), BQ086953 (alpha-tubulin) were used for microgravity versus 1-*g* comparisons (Table 2.4). Experimental genes used were CV735302, CV735900, CV735690, CV735658, BQ087432, BQ087231, and BE642826. Primer sequences for each experimental gene are listed (Table 3.1).

RESULTS

Microgravity versus 1-*g* gene expression

BAGEL analysis of array data requires at least one common sample to compare array hybridizations results. We were most interested in the differences in flight samples as compared to ground. Microgravity spore material was limited, so array analysis of differences between time points within microgravity growth conditions was not possible. Therefore,

changes in abundance over time in microgravity growth conditions can only be made as a comparison to the changes observed in normal 1-*g* development as presented in chapter 2.

Differences in flight and ground samples were analyzed separately for each time-point after light exposure; 1 h, 8 h, and 20 h. Four biological replicas of each comparison were included in BAGEL analysis for each time-point comparison, and the numbers of array features analyzed and percent of those features showing significant differences at each time point were complied (Table 3.2). Many different spots printed on these arrays represent the same *Ceratopteris richardii* tentative unique gene (TUG), so they show that same changes in abundance in this study.

A complete list of microarray spots that have significant differences in abundance between flight and ground for at least one time-point analyzed is presented (Supplemental Data Table 3.3). The putative identity of these TUGs is based on the sequence similarity obtained as described (Chapter 2 Methods). TUGs with significant BLASTX matches have been put into general categories based on the biochemical processes in which the products of these genes are involved (Supplemental Data Table 3.4). Placement of TUGs into these categories was done based on literature review of all data available for a particular gene and cannot be considered a definitive characterization of the *Ceratopteris* genes presented.

However, these categories can be informative about spore cellular processes altered by development in microgravity, much like "clustering" of larger array data sets (Kimbrough et al., 2004).

Comparison of Microarray Data to Quantitative Real-Time RT-PCR

Relative expression levels as determined by microarray analysis were corroborated by quantitative real-time RT-PCR (Q RT-PCR). Three different biological samples were used for each comparison when possible. For some samples there was only enough total RNA for two biological samples to be used. Q RT-PCR was done for seven TUGs that showed significant differences between microgravity and ground samples in microarray analysis. Two different control genes were used. Lack of detectable signal in mock RT controls confirmed the absences of genomic DNA contamination. Fold-change was determined as described in Chapter 2. Mean fold-changes observed by Q RT-PCR using each control gene and by microarray analysis for each of these experimental genes are presented (Fig. 3.1–3.4). Mean differences observed by Q RT-PCR were tested for significant deviations from 0 with a 1-sample *t* -test. Differences were considered statistically significant ($p>0.05$). In all cases when significant difference was determined by microarray BAGEL analysis, Q RT-PCR results using both control genes showed similar expression trends;

moreover, in most cases these differences were statistically significant (Fig. 3.1–3.4).

DISCUSSION

Effects of Space Conditions on Spore Development

The greatly reduced force of gravity is by no means the only difference of the space environment compared to earth. The absence of convection in space results in significantly reduced atmospheric mixing and can cause localized anoxia, or lack of oxygen, that impacts plant growth and development in space (Briarty and Maher, 2004) Although the space flown spores used in this study were kept in the pressurized cabin of the Shuttle, the Petri dishes containing spores were sealed with Parafilm and kept in BRIC containers, so the lack of convection in space might have caused localized anoxia effects on spores, while normal convection in the Petri dishes of the ground controls would have obviated this stress. Over a dozen of the *Ceratopteris* genes that have differential expression induced by spaceflight have significant similarity to genes known to encode enzymes involved in general cellular metabolism (Supplemental Table 3.4). Expression changes of these genes, and possibly others, induced by spaceflight may be indicative of localized anoxia effects on spore development. These effects were not, however, significant enough to

result in obvious differences in spore germination rates or morphology of gametophytes (Chatterjee and Roux, unpublished observations).

Because plants evolved in the earth's gravitational field, the lack of this force may be considered a stress by plants. Consideration of spaceflight as a stress on plant development, and ways to study this have been addressed by Paul and Ferl (2002). A common theme in plant stress responses, abiotic and pathogen attack is an increase in reactive oxygen species (ROS), such as H_2O_2 (Mahalingam and Fedoroff, 2003). Peroxidase is an enzyme that oxidizes various substrates at the expense of H_2O_2, thereby removing this ROS. Decreased peroxidase activity was previously observed in response to microgravity development in *Brassica napus* protoplast cells (Skagen and Iversen, 2000). We have corroborated this observation by the finding that three different *Ceratopteris* genes likely to encode peroxidases are down regulated 1.5 to 2.5 fold in spaceflight development (Supplemental Table 3.4 and Fig. 3.3).

Evaluation of Genes Previously Implicated in Polar Development or Gravity Responses

Several of the *Ceratopteris* genes printed on these microarrays have a known role in either polar development or a gravity response in other model systems. Many genes involved in development and in environmental responses are conserved among all plants evaluated (e.g.

phytochrome) and even across kingdoms (e.g. calmodulin). It follows that genes discovered to be involved in polar and gravity-directed development in other organisms warrant evaluation for their possible role in these processes in *Ceratopteris*. Here we carry out this evaluation.

There have been very few reports on microarray analysis of spaceflight material: no other plant systems, and only a few animal or animal cell arrays. One evaluation of expression changes induced by spaceflight was made on human leukemic T lymphocytes (Lewis et al., 2001). This report focused on the up-regulation, in space compared to ground samples, of 11 genes that encode cytoskeletal proteins. Based on these findings I searched all the genes whose expression in microgravity was determined for BLAST matches to the following proteins or protein families; calponin, dynactin, tropomodulin, keratin, myosin, ankyrin, actin, plectin, centriole-associated, profiling and tubulin. Of the genes in the *Ceratopteris* ESTs encoding these cytoskeletal proteins, the only one that had a significant change between ground and spaceflight samples was likely to encode tubulin beta-8 chain (Supplemental Table 3.4, *Ceratopteris* Accession CV735909). This beta tubulin gene is down-regulated 50% in spaceflight samples 20 h after illumination. The findings in human cultured cells of significant cytoskeletal gene up-regulation were clearly not mimicked in *Ceratopteris* spores in spaceflight.

To identify the genes involved in the response of *Arabidopsis* roots to gravitropic stimulation on earth, two groups have conducted microarray experiments using oligonucleotide arrays (Kimbrough et al., 2004; Moseyko et al., 2002). The changes in gene expression induced by spaceflight reported here have been compared to the finding of Kimbrough et al. (2004) (Table 3.3). This group attempted to differentiate between the gravity-responsive changes in gene expression and those changes induced simply by the movement associated with gravity stimulating the plants. In their experiment, *Arabidopsis* seedlings were grown horizontally on plates, then either rotated 135° then kept in this new orientation, or gently moved back and forth for 5 seconds then kept stationary. They removed the root tips of seedlings at various time points after each treatment, and gene expression changes were evaluated.

There are 14 unique genes that have a change in abundance in spores during spaceflight and also have a change in abundance after gravity stimulation of *Arabidopsis* roots (Table 3.3). The authors suggest that genes with expression changes after both gravity and mechanical stimulation may not be responding specifically to the gravity stimulus, but allow for the possibility that these genes may help regulate responses to both gravity and mechanical stimulation. Not surprisingly, the suppression or induction of these genes is not always the same in spores

and roots. This is the case for a gene predicted to encode CER1, which in spores is down regulated at all time points tested in spaceflight and in *Arabidopsis* roots is up regulated after mechanical and gravity stimulation. These 14 genes have now been implicated in the gravity response of two different plant systems, and their possible roles in gravity signaling definitely need further investigation.

A possible role for RNA localization in the gravity-directed polarization of spores was suggested by the expression profile of a gene likely to encode a *Ceratopteris* Mago nashi protein during the first 48 h of spore development (Chapter 2, Figure 2.5A). If Mago nashi genes are in fact involved in spore gravity response, one might expect to see a difference of expression in microgravity. Interestingly, both of the *Ceratopteris* genes likely to encode Mago nashi proteins have no significant difference in abundance between microgravity and 1-*g* samples at any time-point analyzed. Of course, this does not eliminate the possibility that control of RNA localization by Mago nashi is part of the process by which gravity directs the polarity of development, as changes in it abundance would not be required for its participation in this process.

The only gene that has been definitively shown to be critical for the early response to gravity in higher plants is the *Arabidopsis* gene called Altered Response to Gravity (*ARG1*). The ARG1 protein is involved in pH

changes in response to gravity stimulation and in auxin redistribution (Boonsirichai et al., 2003). The presence of a *Ceratopteris* gene likely to encode ARG1 (accession BE641856) is in itself exciting. Unfortunately, this gene could not be analyzed in the microarray analysis of microgravity-induced changes in expression due to inadequate spot hybridization. Because of the role of this gene in *Arabidopsis* early response to gravity there should be a continued study of this putative *Ceratopteris* ARG including real time RT-PCR analysis of its expression in microgravity.

A change in cytoplasmic pH observed in *Arabidopsis* cells is one of the earliest responses to a gravitropic stimulus (Scott and Allen, 1999; Fasano et al., 2001). This plus the fact that *ARG1* is expressed in spores during the period when gravity is directing their polarity may be related to the finding that in microgravity there is an increased abundance of the mRNAs encoding three proteins that are likely involved in alkalinization of the cytoplasm (Supplemental Table 3.4 accession CV734831, CV735194, and CV735685). Two of these proteins are likely vacuolar-localized proton pumps, and the other is a sodium/hydrogen antiporter. Taken together these data support a rationale for further testing of the role of *ARG1* and other genes encoding proteins that control cytoplasmic alkalinization in the gravity response in *Ceratopteris*.

Among the many genes involved in planar polarity development in animal epithelial cells, a human protein phosphatase 2C has been shown to be a positive regulator of this process by de-phosphorylating and thereby inhibiting a protein (Axin) that inhibits transcription in the *Wnt* signaling cascade (Strovel et al., 2000). In *Ceratopteris* spores developing in microgravity, a gene likely to encode a protein phosphatase 2C (accession CV735645) is up-regulated after 1 h, 8 h, and 20 h of development in microgravity (Supplemental Table 3.4). Because the brassinosteroid signaling pathway in plants has many parallels to the Wnt signaling pathway in animals (Yin et al., 2002), it is possible that this protein phosphatase 2C may play a role in polar growth control in fern spores as in epithelial cells.

Calcium Signaling and *Ceratopteris richardii* Spore Gravity Perception

Calcium acts as a second messenger in many different cellular responses to external stimuli including touch (Legue et al., 1997) and light (Shacklock et al., 1992). Calcium-mediated signaling often functions through release of Ca^{2+} ions into the cytoplasm which binds to any number of Ca^{2+}-binding proteins and either activates or inactivates them. Calcium-mediated signaling of gravity perception in diverse plant cells, including *Ceratopteris* spores, has been suggested frequently in the literature

(Klymchuk et al., 2001; Plieth and Trewavas, 2002; reviewed in Kordyum, 2003). Evidence in spores includes an efflux of Ca^{2+} from the top of germinating spores during their period of gravity-directed polarity determination (Chatterjee et al., 2000). When cells are treated with the Ca^{2+} channel blocker nifedipine, the spore primary rhizoids grow in random orientations (Chatterjee et al., 2000).

These findings indicate that intracellular changes in Ca^{2+} are likely involved in the cellular events of spore gravity signal transduction. Increases in cellular $[Ca^{2+}]$cyt frequently increase the expression of genes for calcium-binding proteins (Braam 1992). Six genes likely to encode calcium-binding proteins have a significant change in abundance between initial light exposure (0 h) and 48 h after light exposure (Supplemental Table 2.5). Five of these genes have their highest expression 48 h after light exposure (Fig. 3.5) and only one, a gene likely to encode a C2 domain protein (Fig. 3.6), appears to have an expression profile coincident with the calcium current observed in developing spores.

The C2 domain is a protein motif that binds phospholipids in a Ca^{2+}-dependent manner (Tomsig et al., 2000). Some proteins involved in vesicle transport have this domain, including synaptotagamin (Earles et al., 2001). *Arabidopsis* phospholipase Dζ, which, like many plant phospholipase D proteins, has a C2 domain (Elias et al., 2002), is involved

in the establishment of planar polarity in epidermal cells that develop into root hairs (Ohashi et al., 2003) by serving as the direct target of the homeobox gene GLABRA2. Its inducible expression promotes ectopic root-hair initiation.

The *Ceratopteris* gene encoding a C2 domain protein is most abundant 12 h after illumination in normal 1-*g* development (Chapter 2, Supplemental Table 2.5) and shows steady decrease in abundance through 48 h after illumination (Fig. 3.6). Expression of this calcium-binding protein is altered by spaceflight. After 8 h of development in microgravity, this *Ceratopteris* gene is up-regulated compared to ground samples (Supplemental Table 3.4 Accession BE642184). These results from cells developing in microgravity and at 1-*g* suggest that the *Ceratopteris* gene for a C2 domain protein might encode a protein involved in coordinating the Ca^{2+} signal with polarized vesicle transport as a very early step in gravity-directed development in *Ceratopteris* spores (see model below).

Lipids and Signaling in Microgravity Development

Differentiating between the direct effect of microgravity on plant development and the secondary effect of anoxia is challenging, but experiments using a spaceflight 1-*g* control have attempted to separate these effects in developing *Arabidopsis* seedlings (Briarty and Maher, 2004). A major finding of this study, which was consistent with several

other spaceflight experiments, is that development in microgravity results in reduced reserve lipid utilization and reduced root growth in *Arabidopsis thaliana,* and since both of these effects are significantly reversed by the 1-*g* control in spaceflight, they cannot be attributed to anoxia alone, but must be due in part to the effects of the lowered gravity stimulus in spaceflight.

The reduced growth of roots in spaceflight may be due in part to significantly reduced utilization of reserve lipids in the cotyledons of seedlings in microgravity. Lipid reserve mobilization is well studied in angiosperm development (Mansfield and Briarty, 1996 and Pfeiffer and Kutschera, 1997), and the use of reserve lipids during the germination of fern spores has long been known (DeMaggio et al., 1980). Our findings of the increased mRNA levels during spaceflight of ten different *Ceratopteris* genes whose products are likely involved in mobilization of reserve lipids (Supplemental Table 3.4 - biochemical process called seed storage protein) suggest that reduced utilization of reserve lipids in microgravity occurs in ferns as well as angiosperms and may, in fact, be universal to all plants.

A cellular feedback mechanism at 1-*g* may inhibit the expression of genes encoding storage proteins when reserve lipids have been depleted. Evidence that expression of at least one of these genes is down regulated during early development was found (Chapter 2 – Supplemental Table 2.5;

Fig.3.7). An alternate explanation of the same data is that in normal 1-*g* development the mRNAs encoding storage proteins are present in dormant spores, but once germination is initiated, the mRNA is translated into the proteins needed by the cell, then destroyed, and the spore does not produce any more of the message. However, if reserves are not being used up, as in microgravity conditions, the inhibition of transcription and/or the turnover of the mRNA for storage proteins may not happen.

All of the other genes for seed storage proteins that have enhanced message levels during development in microgravity either show no significant change during early development at 1-*g* or were not analyzed in that experiment. One *Ceratopteris* gene whose product is likely involved in mobilization of storage lipids is down-regulated by microgravity development 8 h after illumination (Supplemental Table 3.4 – Accession BE640941), indicating that in this proposed mechanism a lack of feedback inhibition may not be accurate for all genes involved in this complex process. Our findings that the levels of mRNAs related to reserve lipid metabolism are increased by microgravity within 8 h after the initiation of germination in *Ceratopteris* are consistent with the finding of increased lipid content 86 h after germination begins in *Arabidopsis*.

The detection of the directional force of gravity by germinating spores is converted into cellular signaling events that mediate polarized

development, including polar cell division. Without the force of gravity, some of the molecular participants of this signaling pathway may not be produced by spores. Observations of spores that developed on the space shuttle show that directional nuclear migration, which predicts cell division asymmetry, as well as asymmetric division and rhizoid growth, all occur in microgravity development, but with randomized directionality (Roux et al., 2003). In this study we found six different *Ceratopteris* genes whose products are likely involved in lipid hydrolysis/synthesis/or transfer that show reduced mRNA levels during development in microgravity (Fig. 3.1 and Supplemental Table 3.4).

The role of lipids in the induction and stabilization of plant cell polarity was recently reviewed (Fischer et al., 2004). Polar sorting of vesicles is critically required for the directional expansion of the root cells in the elongation zone of *Arabidopsis*, and there is evidence that this polar sorting of vesicles is mediated by GPI-anchored proteins (GAP) in the plasma membrane. The *Arabidopsis* protein AIR12, which accumulates during auxin induced lateral root formation (Neuteboom et al., 1999) is a GAP (Borner et al., 2002), and a *Ceratopteris* gene with high sequence similarity to AIR12 is up-regulated after 1 h of development in microgravity (Supplemental Table 3.4). If this GAP is also important for

directional growth in *Ceratopteris,* suppressing its expression could block or disorient the normal polar development in spores.

Many *Arabidopsis* lipid transfer proteins have been shown to be GAPs (Borner et al., 2002; Elortza et al., 2003). Our finding that during development in microgravity there is about a 3-fold decrease in expression of a gene likely to encode a lipid transfer protein (Fig. 3.1) as well as decreased expression of five other genes whose products are possibly GAPs (Supplemental Table 3.4) suggests that these gene products might be involved in polar vesicle sorting to the plasma membrane after cellular detection of gravity. Further analysis into this process of localized vesicle transport mediated by GPI-anchored proteins may help elucidate the earliest cellular events in response to gravity perception.

Table 3.1 Real Time Quantitative RT-PCR Primers.

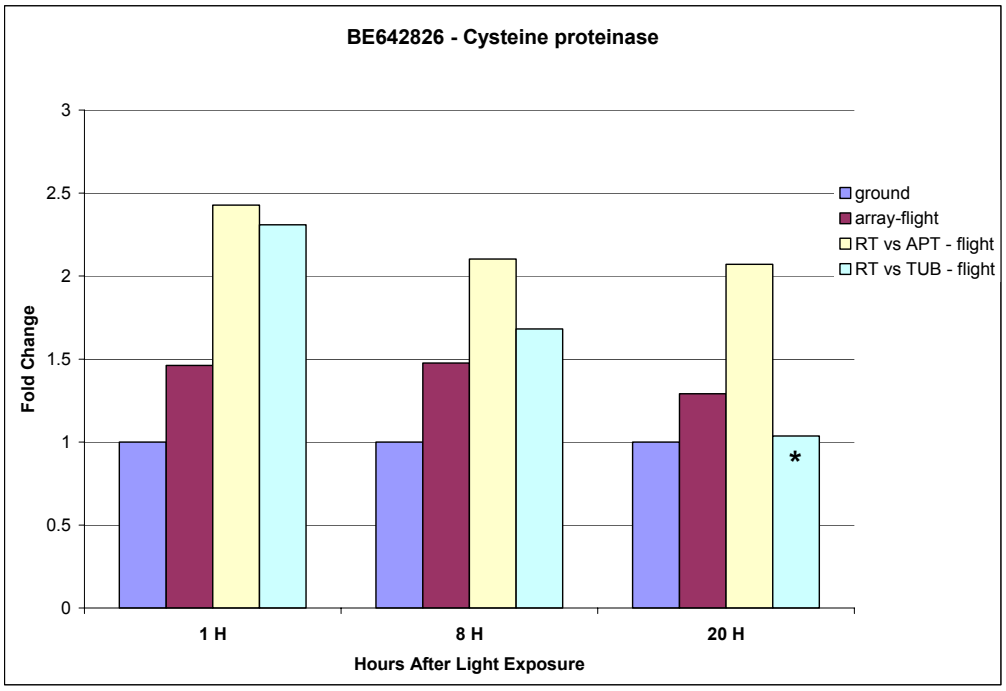
Target	Labeled forward primer	Unlabeled reverse primer
BE642826	GAACGGGAGCTTGCTGGGCG[FAM]TC	GTTTCCGGTCGCCACGAAGT
BQ087231	CACTTTACGCATCCGTCCAAAG[FAM]G	AATCATAGGCTTGTCGCCAGGT
BQ087432	CACAAACGGATCGGAGCTATGGAGTTTG[FAM]G	CGATTACAGGAGGCGTCGAGA
CV735658	GACATTCTGCGGCGACTGGAAATG[FAM]C	CGGCGGAACGACTAAACAGG
CV735690	CAAACTCTAGCAAGGAAGCGGAGGAGT[FAM]TG	GTCCTTTGCGGAGGAGATGTG
CV734892	GAACGATGACATCTCCAACTCCTTTCG[FAM]TC	CACACGATCCTTTGCCTCCAT
CV735302	GACGAGCAATGACCGCACTAACCTCG[FAM]C	GCGGCCAAATCGCTGAAATA

Table 3.2 Statistical analysis of microgravity microarray results. Each time-point of development in microgravity was evaluated separately in four replicated microarrays. Only array features that met quality criteria standards in at least three replicas of any array comparison were analyzed for significant changes between ground and microgravity at that developmental time-point.

	Number of spots analyzed	% Spots altered by flight	Unique genes up-regulated by flight	Unique genes down-regulated by flight
1 H	2,110	3.46%	37	25
8 H	2,486	5.91%	70	42
20 H	2,797	5.20%	53	58

Table 3.3 Genes involved in gravity or mechanical stimulation of *Arabidopsis* roots and *Ceratopteris* spore gravity response.

Gene Name	TAIR ID	*Ceratopteris* Accession	*Ceratopteris* Expression in Microgravity	*Arabidopsis* Expression after Gravity or Mechanical Stimulation
catalase 3	At1g20620	CV735670	Up after 8 h	Up After Gravity stimulation
CHP-rich zinc finger protein, putative	At5g43030	CV734713	Up after 20 h	Up after both
senescence-associated protein, putative	At4g28050	CV734917	Up after 20 h	Up after both
fibrillarin 2 (AtFib2)	At4g25630	CV735883	Up after 20 h	Up after both
glycerol-3-phosphate dehydrogenase, putative	At3g10370	BE640862	Up after 20 h	Up after both
S-adenosyl-methionine-sterol-C-methyltransferase -related	At1g76090	BE640989	Up after 20 h	Up after both
mitochondrial carrier protein family	At1g74240	BE642858	Up after 8 h	Up after both
hypothetical protein	At1g72480	CV735059	Up after 1 h	Up after both
protodermal factor 1	At2g42840	CV734892	Down after 8 and 20 h	Down after both
DNA-binding protein -related	At3g61260	BE642870	Down after 20 h	Down after both
In2-1 protein, putative	At3g55040	CV734816	Up after 8 h	Down after both
nucleolar protein Nop56, putative	At3g12860	CV734993	Down after 1 h	Up after both
thioredoxin family	At1g53300	CV735114	Down after 20 h	Up after both
CER1 protein, putative	At5g57800	CV735081	Down after 1, 8, and 20 h	Up after both



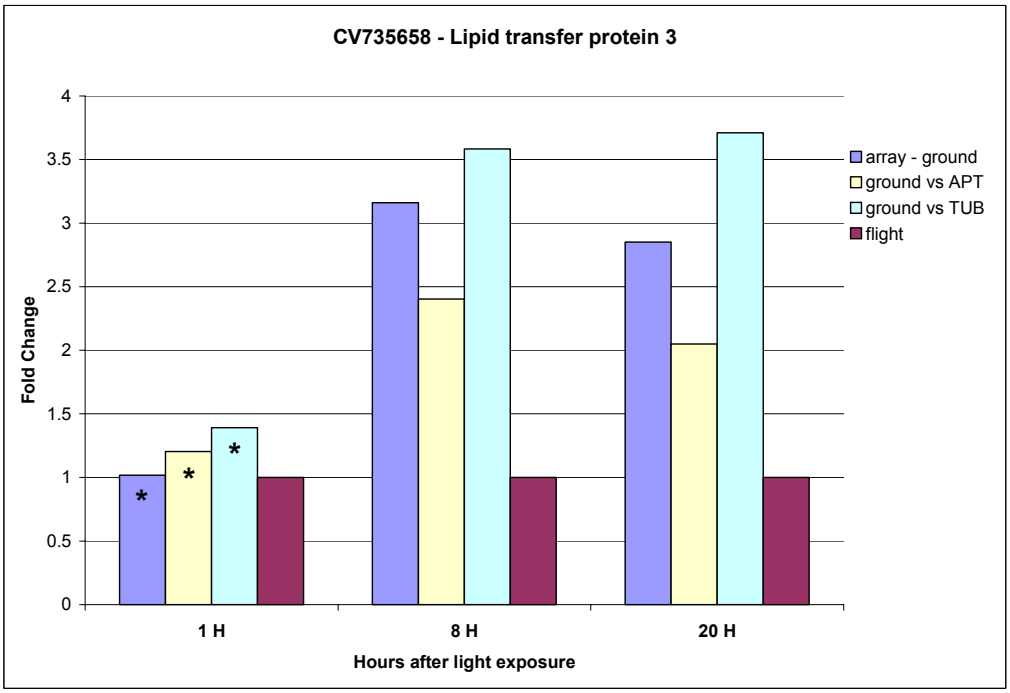
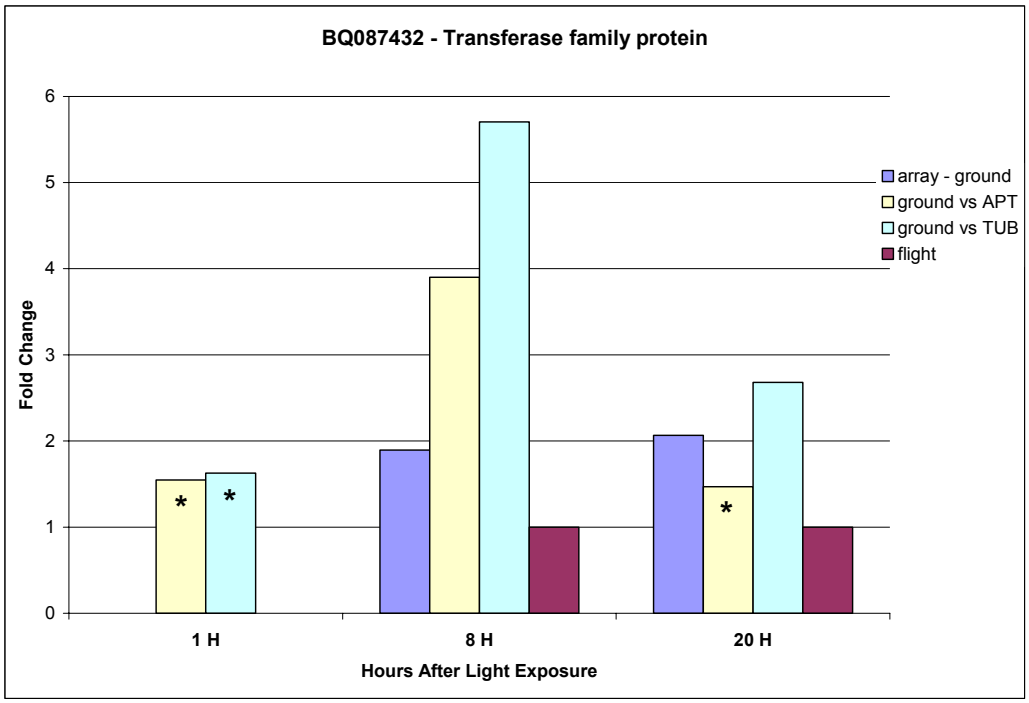


Figure 3.1 Comparison of microarray and Q RT-PCR analysis of gene expression. Genes are identified by accession and highest BLASTX match identity. All fold changes are statistically different from 1 unless indicated by asterix (*).



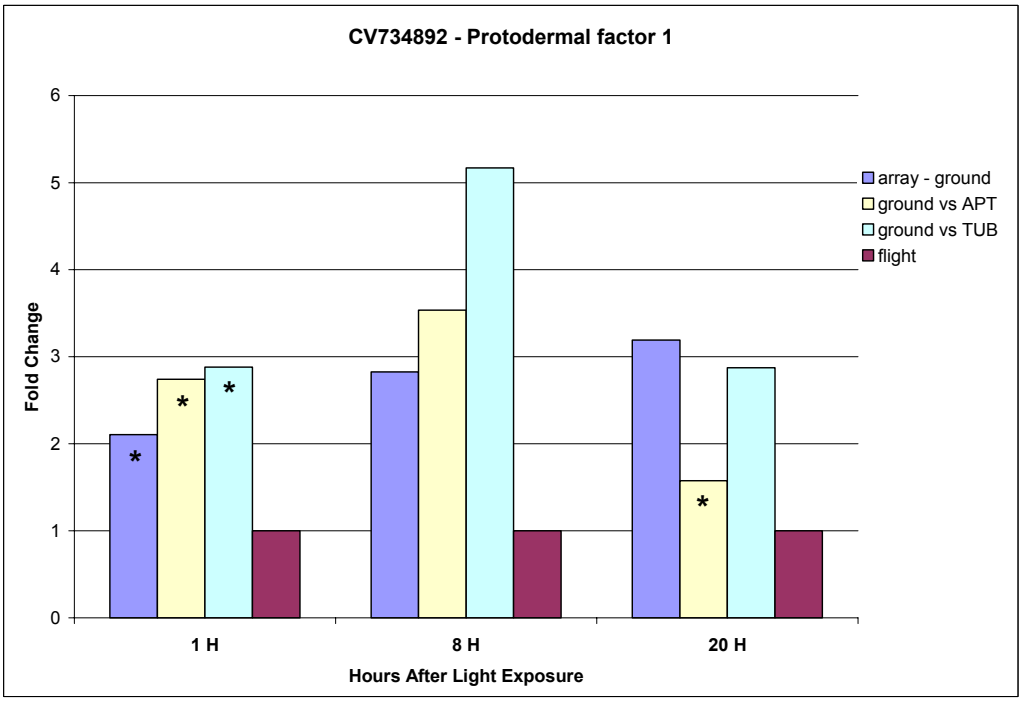
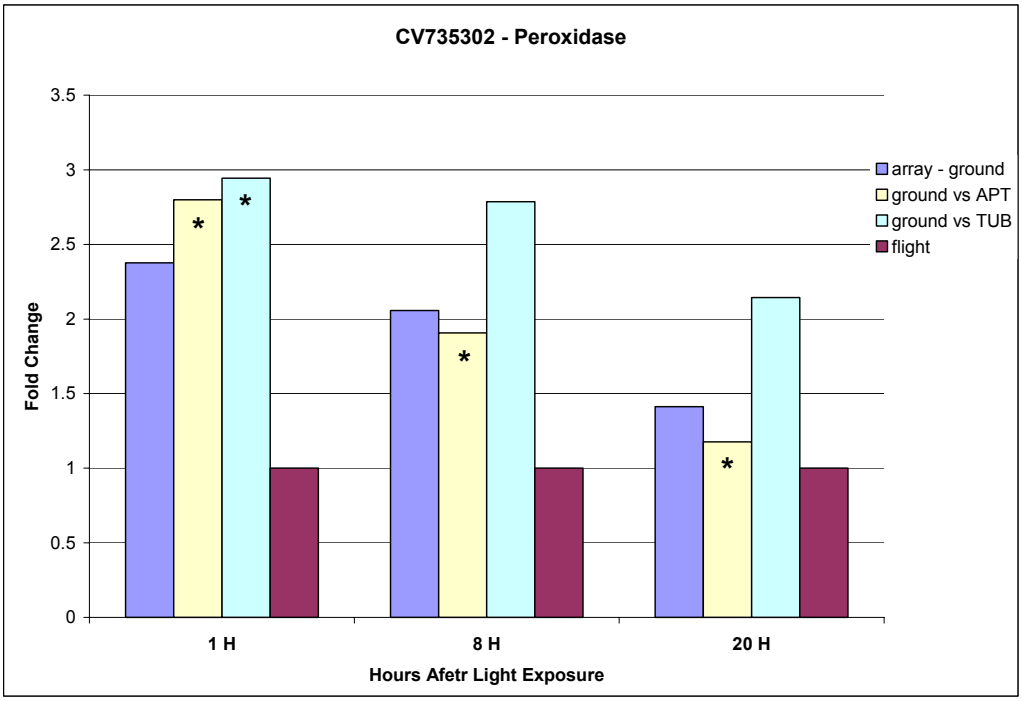


Figure 3.2 Comparison of microarray and Q RT-PCR analysis of gene expression. Genes are identified by accession and highest BLASTX match identity. All fold changes are statistically different from 1 unless indicated by asterix (*).



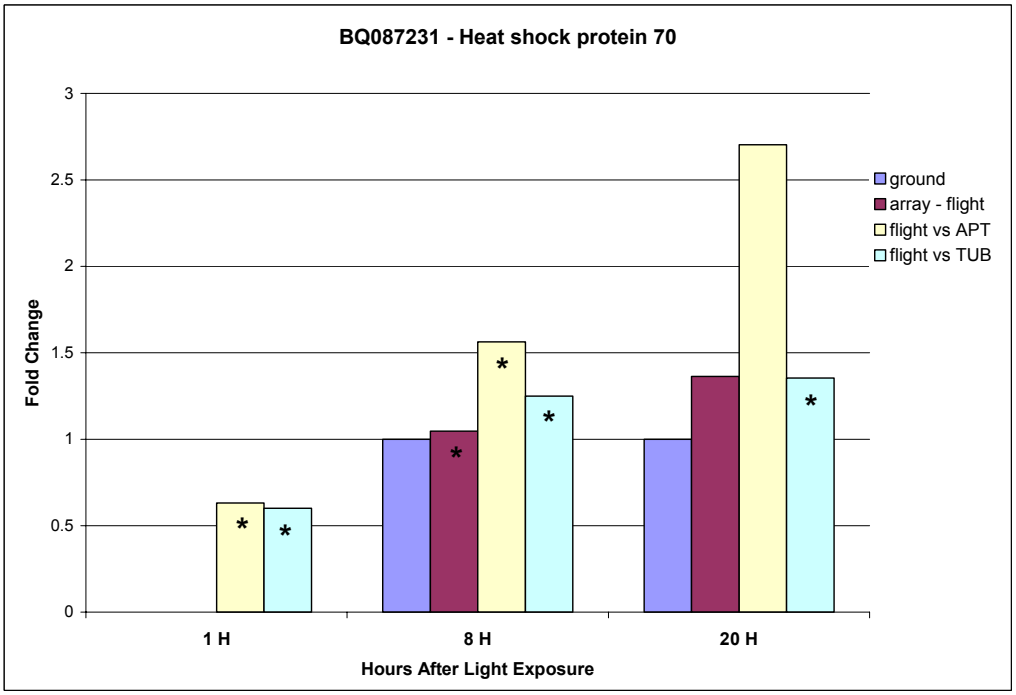


Figure 3.3 Comparison of microarray and Q RT-PCR analysis of gene expression. Genes are identified by accession and highest BLASTX match identity. All fold changes are statistically different from 1 unless indicated by asterix (*).

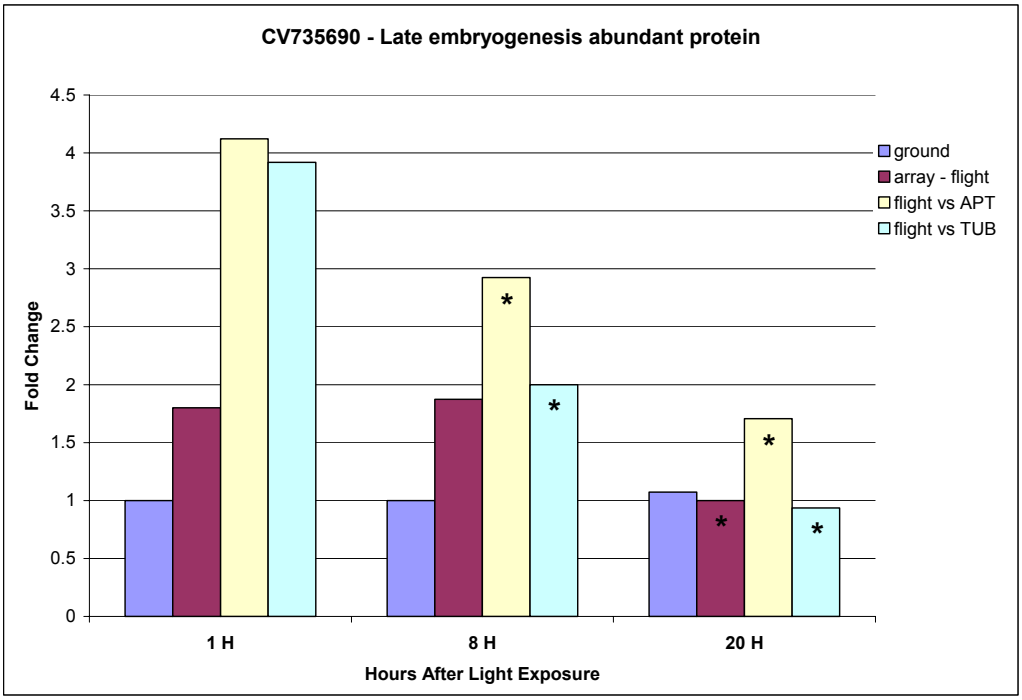


Figure 3.4 Comparison of microarray and Q RT-PCR analysis of gene expression. Genes are identified by accession and highest BLASTX match identity. All fold changes are statistically different from 1 unless indicated by asterix (*).

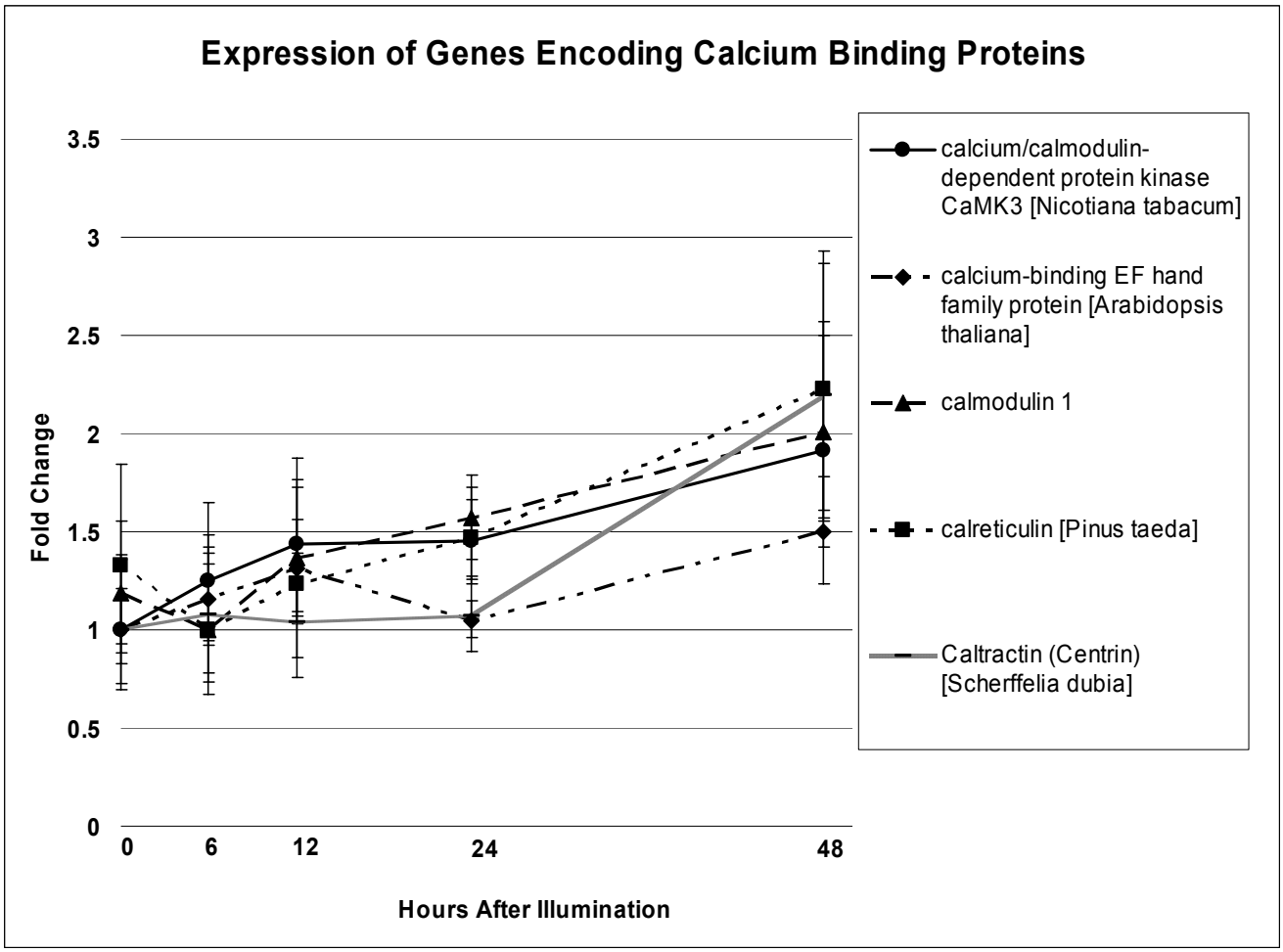


Figure 3.5 Expression Profiles of Genes Encoding Calcium Binding Proteins. The mRNA levels for all 5 of these genes likely to encode different calcium binding proteins are significantly up-regulated during the first 48 h of spore development. Error bars represent 95% credible interval of fold changes compare to 1, based on BAGEL analysis of replicated array analyses.

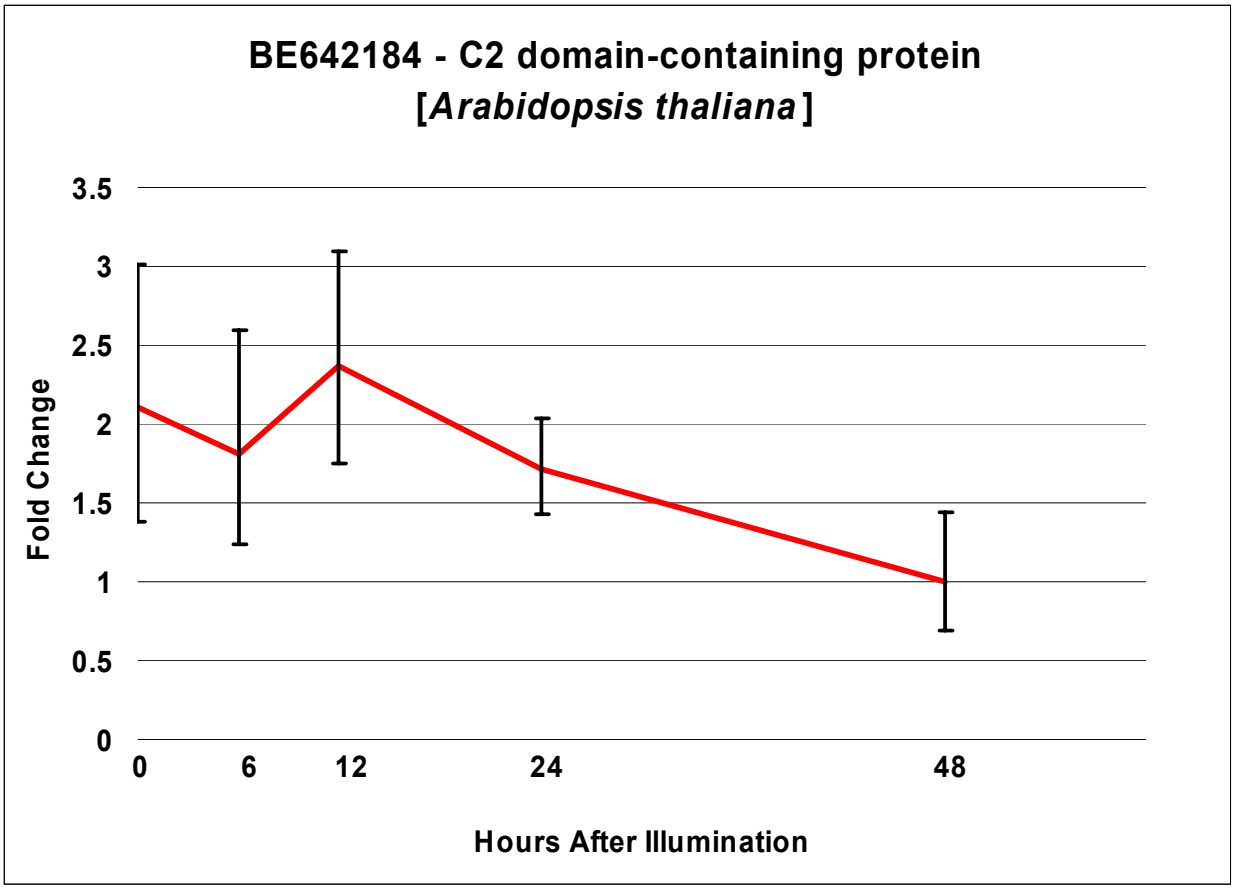


Figure 3.6 Down regulation of the abundance of mRNA for C2-domain-containing protein during the first 48 h of spore development. Error bars represent 95% credible interval of fold changes compare to 1, based on BAGEL analysis of replicated array analyses. Two fold decrease in abundance of this *Ceratopteris* gene from between the time of illumination (0 h) and 48 h after illumination is significant based on non-overlapping credible interval.

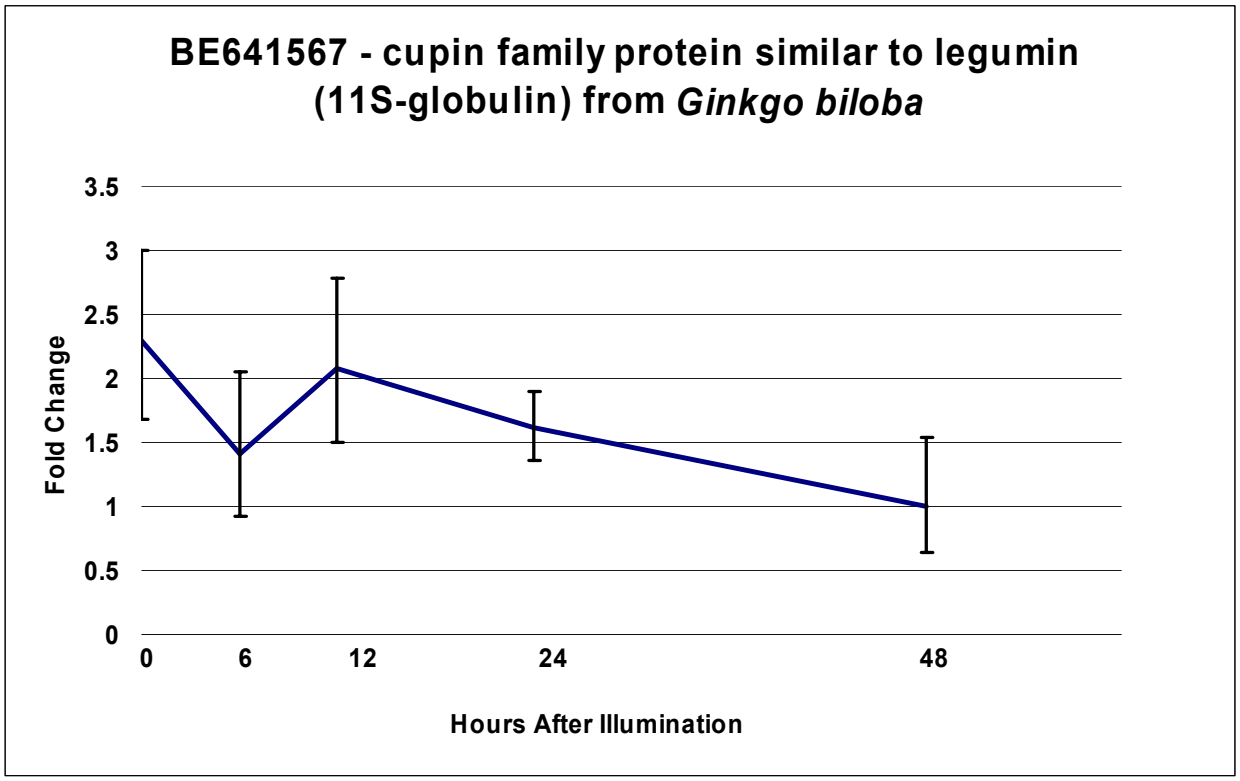


Figure 3.7 Down regulation of abundance of mRNA for cupin family protein during the first 48 h of spore development. Error bars represent 95% credible interval of fold changes compare to 1, based on BAGEL analysis of replicated array analyses. Two fold decrease in abundance of this *Ceratopteris* gene from between the time of illumination (0 h) and 48 h after illumination is significant based on non-overlapping credible interval.

CHAPTER 4

CONCLUSION

The unifying theme of the data presented here is documentation of the gene changes involved in the early development of *Ceratopteris* gametophytes, including the earliest signaling steps of the spore's response to gravity, and discussion of their significance. Detailed analysis of expression changes during spore emergence from dormancy (presented in Chapter 2) is particularly interesting in light of the similarity of genes expressed during germination in fern spores and angiosperm seeds. The large-scale analyses presented here of gene expression changes induced by the spaceflight environment during the early development of a plant are, to our knowledge, the first of their kind. The extreme rarity of spaceflight experimentation since the Space Shuttle Columbia disaster in 2003 makes these findings particularly valuable.

Despite completion of the genome sequencing of *Arabidopsis* and rice, and the availability of numerous whole-genome microarrays for *Arabidopsis*, there are currently no reports of microarray analysis of the global changes in mRNA abundance that occur during germination of any seed. This type of analysis is no doubt technically challenging, as array

hybridization requires large amounts of RNA; and obtaining high quality, non-degraded RNA from seeds, as with spores, is difficult. Our evaluation of the spore ESTs demonstrates the somewhat unexpected similarity of genes expressed in spores and seeds, and spores and pollen. When analysis of the global expression changes that occur during seed emergence from dormancy becomes available, comparison of spore and seed developmental gene expression will be very useful in considering the evolutionary origins of the seed.

The profile of gene expression changes during the early development of germinating spores provides clues as to possible molecular mechanisms of breaking dormancy implemented by plant cells. The subset of genes up- and down-regulated just prior to the first cell division of spores may reveal new participants in that critical process and should continue to be studied.

Microarray analysis of the effect of spaceflight on plant gene expression was suggested to be a necessary and obvious step in understanding plant responses to space growth conditions (Paul and Ferl, 2002). The data presented here (Chapter 3) contribute significantly to our understanding of how the microgravity environment impacts the nuclear metabolism of cells and of what signaling events may transduce the force

of gravity into developmental changes in spores and possibly other cell types.

The inclusion of a 1-*g* space-flight control in this experiment would have allowed us to make very clear distinctions between the effect of microgravity and that of other stresses due to the space environment. That option was not available for the STS-93 Shuttle flight. Without such a control I can only interpret these findings in light of previous studies of the physiological changes induced by space-flight in other plants. As is, our analysis of spaceflight-induced expression changes in spores during the period of their polarization will serve as a valuable information base that can be integrated with the results of future spaceflight studies to generate a more complete understanding of the perception and response of cells to the microgravity environment.

Prior to this work, our model of spore gravity response predicted that the perception of gravity leads to a trans-cell calcium gradient driven by the entry of calcium along the bottom of the cell and its localized efflux from the top of the spore through the activation of Ca^{2+} pumps at this location. Intracellular Ca^{2+} gradients are known to enhance cytoskeleton rearrangements and polarized vesicle transport preferentially to the site of calcium entry, as repeatedly demonstrated in polarly-growing pollen tubes

(Wang et al., 2004), algae rhizoids (Braun et al., 2004), and root hairs (Shaw and Long, 2003). Possibly related to this observation is our evidence that the mRNA for a gene encoding a C2 domain-containing protein is most abundant coincident with the calcium current in 1-*g* development and is up-regulated after 8 h of microgravity development. However, to test the importance for cell polarization of this gene and any of the other genes whose mRNA levels change dramatically during gravity-directed polar development, a reliable method of knocking out spore-expressed genes is needed.

Suppression of gene expression by bombardment with double stranded RNA has been demonstrated in mature gametophytes, and introduction of exogenous antisense RNA to germinating spores has also been successful. These techniques and others for inhibiting gene expression will have to be tested in order to develop an optimized method for suppressing spore-expressed genes.

The microarrays described here contain an estimated 25% of genes expressed in germinating spores. Sequencing more ESTs from the spore cDNA library and their addition to the microarray no doubt would increase the usefulness of this microarray and provide a more complete picture of spore gene expression. Despite the limitation of the current

microarray, the ability to monitor over 2,000 genes simultaneously has been informative and has allowed me to find possible participants in spore gravity response of which there was no pre-conceived expectation.

The microarray is also being used to evaluate the role of nitric oxide signaling in spore gravity perception and response in collaboration with the Porterfield lab. The gene expression profile of spores in which the response to gravity has been altered by conditions other than microgravity, such as nifedipine treatment (Chatterjee et al., 200) and treatment by nitric oxide-inhibiting drugs (data not shown), should be evaluated. Comparison of these findings to the profile of message level changes that occur in spores that develop in microgravity will help to clarify the mechanism of the spore's response to gravity.

Large scale analysis of expression changes, like those described here, can be helpful at identifying specific genes or biochemical processes that may be involved in cellular signaling pathways because it is not dependent on predictions about genes involved in the response. This methodology can also be useful in examining processes and genes previously implicated in a response. Levels of gene expression may not, however, be paralleled by protein expression. To address this question, researchers in the Roux lab have obtained specific antibodies for three

different proteins involved in calcium signaling (Stout, 2004; Bushart personal communication). They used these antibodies in Western Blot analyses that showed changes in the abundance of all three proteins mimicked the changes in abundance of the mRNA for these genes.

Microarray analysis is a relatively new technique. Generally accepted standards and methods of analysis of the large data sets obtained are still being developed. As with any experimental approach, the reliability of microarray data is dependant on the amount of replication conducted. For this reason, the array comparisons presented here were replicated a minimum of four times. Due to the inherent variability between microarray hybridizations and the large amount of information produced, comparisons of replicated experiments require computation software specific to this task. The software chosen for this dissertation, BAGEL, requires at least three replicas of any comparison made, and evaluates the statistical reproducibility of the expression levels observed. This level of replication and statistical analysis demonstrate the reliability of the data presented here.

The utility of the microarray constructed in this project has been verified by two separate evaluations of expression changes induced by different ways: changes that occur over time during normal development,

and changes induced by microgravity. We expect that the results of these analyses will be very useful, both to other researchers using the *Ceratopteris* spore to study different aspects of plant cell development, and, more broadly, to biologists studying cell polarization and the response of cells to gravity.

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